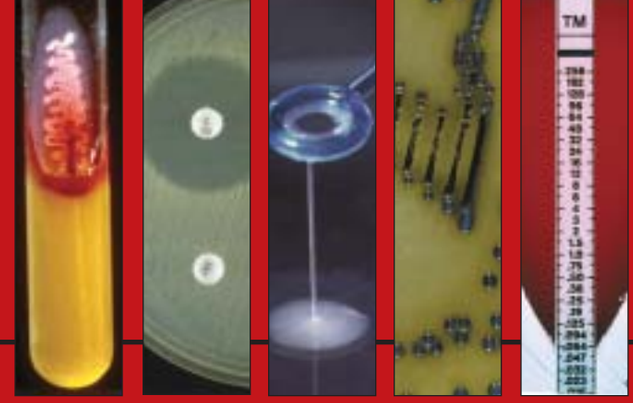

WHO/CDS/CSR/RMD/2003.6

Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World

Haemophilus influenzae,
Neisseria meningitidis,
Streptococcus pneumoniae,
Neisseria gonorrhoeae,
Salmonella serotype Typhi,
Shigella, and
Vibrio cholerae

WORLD HEALTH ORGANIZATION

Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World

Haemophilus influenzae, Neisseria meningitidis,
Streptococcus pneumoniae,
Neisseria gonorrhoeae, Salmonella serotype Typhi,
Shigella, and *Vibrio cholerae*

Principal Authors

Mindy J. Perilla, MPH
Gloria Ajello, MS—*Haemophilus influenzae* and *Neisseria meningitidis*
Cheryl Bopp, MS—*Salmonella* serotype Typhi, *Shigella*, and *Vibrio cholerae*
John Elliott, PhD—*Haemophilus influenzae* and *Streptococcus pneumoniae*
Richard Facklam, PhD—*Haemophilus influenzae* and *Streptococcus pneumoniae*
Joan S. Knapp, PhD—*Neisseria gonorrhoeae*
Tanja Popovic, MD PhD—*Haemophilus influenza*e and *Neisseria meningitidis*
Joy Wells, MS—*Salmonella* serotype Typhi, *Shigella,* and *Vibrio cholerae*
Scott F. Dowell, MD MPH

Centers for Disease Control and Prevention, Atlanta, Georgia, USA

This manual was prepared by:
Centers for Disease Control and Prevention: National Center for Infectious Diseases
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Additional copies of this book can be obtained from either:—

Centers for Disease Control and Prevention
Respiratory Diseases Branch
Division of Bacterial and Mycotic Diseases
National Center for Infectious Diseases
Centers for Disease Control and Prevention
1600 Clifton Road, NE MailStop C-23
Atlanta, Georgia 30333 USA
Fax: (+1) 404 639 3970

or: –

CDS Information Resource Centre
World Health Organization
1211 Geneva 27
Switzerland
Fax: (+ 41) 22 791 4285
E-mail: cdsdoc@who.int

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CDC
Ron Ballard
Melinda Bronsdon
James Gathany
Christopher Jambois
Leslye LaClaire
Lynne McIntyre
Eric Mintz
Susanna Schmink
Anne Schuchat
Benjamin Schwartz
Yvonne Stifel
J. Todd Weber

USAID
Andrew Clements
Anthony Boni
Deborah Lans
Michael Zeilinger
Robert S. Pond (USAID/Ghana)

WHO
Bradford A. Kay (WHO/AFRO)
Rosamund Williams (WHO)
Philip Jenkins (WHO)
Claus C. Heuck (WHO)
Antoine Kabore (WHO/AFRO)
Wondi Alemu (WHO/AFRO)
Claire-Lise Chaignat (WHO)

South African Institute for Medical Research
Keith A. Klugman
Robin Huebner
Anne von Gottberg

Zimbabwe National Public Health Laboratory
Bekithembga Mhlanga
Vladanka Rasevski
Eileen Burke (DANIDA)
Munyaradzi Chipfupa
Alexander Dzapasi
Monica Kureva
Owen Tafirenyika Mandisodza
Joshua Mandozana
Maqhawe Ndlovu
Gladys Nyamimba
Lazarus Manenji Zawaira

Noguchi Memorial Institute for Medical Research—Ghana
David Ofori-Adjei
Patience Akpedonu
Kwaku Owusu-Darko

Komfo Anokye Teaching Hospital—Kumasi, Ghana
Ohene Adjei

University of Ghana Medical School—Accra, Ghana
Mercy Newman

Tropical Diseases Research Centre—Zambia
Mathias Tembo

Danish Veterinary Laboratory—Copenhagen, Denmark

International Centre for Diarrhoeal Disease Research, Bangladesh
G. Balakrish Nair

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Bacterial Agents of Enteric Diseases of Public Health Concern

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List of Abbreviations Used in This Document

APW	Alkaline peptone water
ASM	American Society for Microbiology
ATCC	American Type Culture Collection
BS	Bismuth sulfite agar
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CSF	Cerebrospinal fluid
CTA	Cystine trypticase agar
DCA	Desoxycholate citrate agar
DE	Dorset egg medium
DGR	Dangerous Goods Regulations (publication)
GC	*Neisseria gonorrhoeae* (or, gonococcus)
GN	Gram-negative broth
HE	Hektoen enteric agar
HIA	Heart infusion agar
Hib	*Haemophilus influenzae* serotype b
HTM	*Haemophilus* test medium
IATA	International Air Transport Association
ICAO	International Civil Aviation Organization
ICG	International Collaboration on Gonococci
KIA	Kligler iron agar
LIA	Lysine iron agar
MAC	MacConkey agar
MIC	Minimal inhibitory concentration
ML	Martin-Lewis medium

MTM	Modified Thayer-Martin medium
NAD	Nicotinamide adenine dinucleotide (V factor)
NCCLS	Formerly known as the "National Committee on Clinical Laboratory Standards," NCCLS is an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.
NP	Nasopharyngeal
PBS	Phosphate buffered saline
QC	Quality control
RCF	Relative centrifugal force (measured in xg)
SEL	Selenite broth
SIM	Sulfide-indole-motility medium
SPS	Sodium polyanetholesulfonate
SS	*Salmonella-Shigella* agar
STGG	Skim-milk tryptone glucose glycerol medium
STI	Sexually transmitted infection
TCBS	Thiosulfate citrate bile salts sucrose agar
T-I	Trans-isolate medium
TSA	Tryptone-based soy agar
TSB	Tryptone-based soy broth
TSI	Triple sugar iron agar
UN	United Nations
WHO	World Health Organization
XLD	Xylose lysine desoxycholate agar

CHAPTER 1

Introduction

Respiratory and enteric diseases comprise a substantial proportion of the burden of morbidity and mortality in the developing world; acute respiratory infection and diarrheal illness are the top two killers of children less than five years of age worldwide. Reproductive tract pathogens cause uncomplicated infections of the mucosal membranes; however, if left untreated, infections with these pathogens can also lead to pelvic inflammatory disease, ectopic pregnancies and infertility, and may facilitate the transmission of HIV. Public health interventions such as access to safe water, improved sanitation, hygiene, immunizations, education, health communication, and access to acute medical care with appropriate case management have contributed to on-going improvements in health, and in social and economic development. One outcome of the increased availability of antimicrobial agents for symptomatic treatment of illness in hospitals and community environments, however, has been the emergence of antimicrobial resistance in pathogens of public health concern.

Antimicrobial resistance is an issue of great significance for public health at the global level. However, it is of particular concern in the developing world because fewer affordable and appropriate treatment options are readily available. It has become increasingly important to monitor patterns of resistance as the antimicrobial susceptibility of bacterial pathogens which contribute significantly to the burden of respiratory, febrile, reproductive tract, and diarrheal illness has declined. Because antimicrobial susceptibility testing is resource-intensive, the World Health Organization (WHO) recommends that only one or two reference laboratories in a country perform these tests. Until now, however, there has not been a technically appropriate source of standardized information for laboratory detection of antimicrobial resistance that is practical for use in regions with limited resources.

This laboratory manual focuses on seven bacterial pathogens of public health importance in the developing world: *Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae, Neisseria gonorrhoeae, Salmonella* serotype Typhi, *Shigella,* and *Vibrio cholerae.* Methods for the isolation and identification of each of these bacterial agents from clinical specimens are presented, and

standardized antimicrobial susceptibility testing techniques and criteria for interpretation are described. To benefit from the information presented in this manual, laboratorians must have received training in proper basic microbiological techniques and be comfortable with such tasks as sterilization of instruments and media preparation. Flow charts of procedures and color figures of bacterial colonies and typical reactions have been provided as supplements to the text for ease of comparative identification. Procedural accuracy and methodological standardization are critical to the performance of antimicrobial susceptibility testing, and adherence to protocols of quality control is also vital to ensure that test results are valid and meaningful.

In order for a laboratory to successfully undertake isolation, identification, and antimicrobial susceptibility testing responsibilities, it must participate in on-going investments in materials, supplies, media, reagents, and quality control, along with periodic training of personnel and quality assessment or proficiency testing. Any deviations from antimicrobial susceptibility testing methods as described in the following pages may invalidate the test results. Antimicrobial susceptibility test methods must be performed as described according to internationally recognized clinical guidelines such as those provided by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis) in order to provide meaningful results for clinical and epidemiological interpretation. Laboratory staff must be afforded the appropriate time and resources to carry out the procedures described in this manual if the results are to be meaningful and applicable to clinical and policy decisions.

As resistance to antimicrobial agents in the pathogens causing these diseases grows and changes, strategies of response also must evolve. Resistant pathogens can translate to fewer treatable infections and thus higher morbidity and mortality, a drain on resources, and an obstacle to social, economic, and health development overall. Timely communication between the laboratory and public health officials is essential to the shaping of locally treatment appropriate policies; the data collected in the laboratory are crucial components of the decision-making process for clinical and public health policies.

Mindy J. Perilla, MPH
Division of Bacterial and Mycotic Diseases
National Center for Infectious Diseases
Centers for Disease Control and Prevention

CHAPTER II

Requirements of a Reference Laboratory

FOR THE PERFORMANCE OF STANDARDIZED ANTIMICROBIAL SUSCEPTIBILITY TESTING

A reference laboratory differs from a clinical laboratory in that microbiologists are able to dedicate their time to confirmation and investigation of isolates sent in from other laboratories or hospitals and (for the purposes of this manual) then perform standardized antimicrobial susceptibility testing. This manual is written and intended for use in a reference laboratory or national central laboratory setting, where material resources are consistently quality controlled and available in sufficient quantities for regular testing of isolates. Reference laboratories must participate in a quality assurance program at least once per year and should also administer quality assurance programs for laboratories in their jurisdiction; the World Health Organization (WHO) encourages central public health laboratories in countries with limited resources to establish national quality assessment schemes and to participate in at least three surveys per year. Time, supplies, and personnel can be costly; as a result, it is anticipated that not every country will be able to support a reference laboratory meeting these requirements. A country that can not establish a reference laboratory should consult a regional or sub-regional reference laboratory for further guidance and for advice on where to send isolates requiring further investigation.

In order to carry out the standardized procedures referred to in this laboratory manual (and many others), the laboratory must be able to make ongoing investments in equipment, supplies, and human resources (*i.e.*, trained laboratorians). The Ministry of Health (or similar appropriate agency) should therefore ensure that its central public health laboratory has the following items of great importance:

- Laboratory space
- Trained laboratory technologists
- Water (purified either by filter system or distillation apparatus)
- Stable source of electricity
- Equipment
  - Water bath
  - Incubator
  - Refrigerator
  - Freezer
  - Autoclave
  - Vortex mixer
  - Labels and/or permanent marking pens
  - Materials for record-keeping (*e.g.,* laboratory log-books, and a computer with printer and Internet / e-mail access)
  - Antimicrobial disks and / or antimicrobial gradient agar diffusion tests (Etests®) (*depending on the organisms to be tested*)
- Standard laboratory supplies (*e.g.,* plates, tubes, pipettes, flasks, inoculating loops, other glassware or plasticware, rulers, bunsen burners or alcohol burners, pH meter, bleach, alcohol), media and reagents

It is also of considerable importance that the reference laboratory have an open line of communication with public health authorities, including ministries of health, professionals in the medical field, and policymakers. If the laboratory is responding to an epidemic that extends across borders, an outside public health agency (*e.g.,* the WHO) may become involved; in such situations, it is significant that data from the laboratory will enable better decision-making for clinical treatment and public health policy in more than one country.

Bacterial Agents of Pneumonia and Meningitis

Haemophilus influenzae
Neisseria meningitidis
Streptococcus pneumoniae

CHAPTER III

Haemophilus influenzae

CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Haemophilus influenzae is a common etiologic agent of diseases such as pneumonia, meningitis, otitis media, and conjunctivitis. Meningitis caused by *H. influenzae* occurs almost exclusively in children less than five years of age, and most invasive *H. influenzae* disease is caused by organisms with the type b polysaccharide capsule (*H. influenzae* type b, commonly abbreviated as Hib). There are conjugate vaccines to prevent *H. influenzae* infections caused by serotype b, though they are not widely available in some parts of the world. No vaccines for the other serotypes or for unencapsulated strains have been developed. Although meningitis is the most severe presentation of disease, *H. influenzae* pneumonia causes more morbidity than *H. influenzae* meningitis.

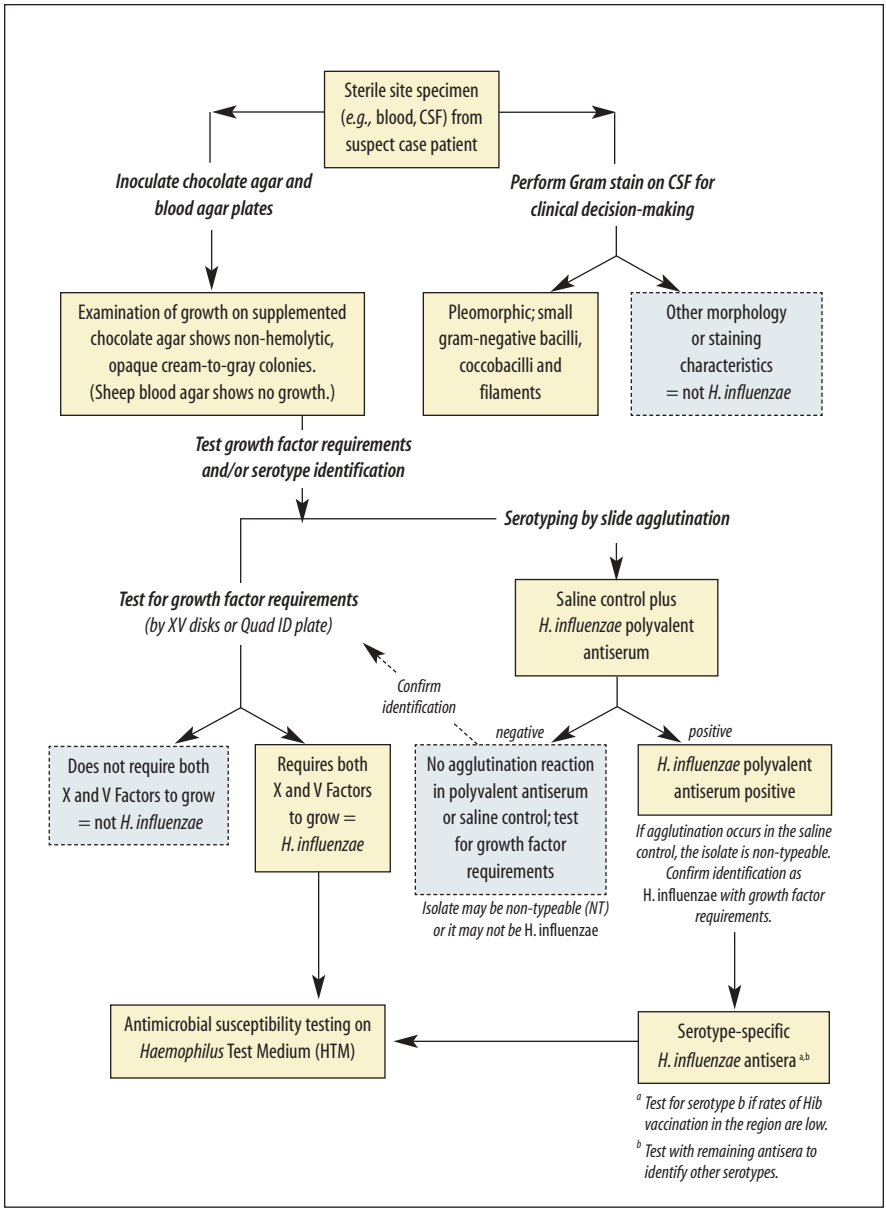
Confirmatory identification of *H. influenzae*

H. influenzae are characterized as small, gram-negative bacilli or coccobacilli that require X and V growth factors, grow on chocolate agar (but **not** on sheep blood agar), and have a pungent indol smell. Methods for the isolation and presumptive identification of *H. influenzae* are included in Appendix 4. Figure 1 presents a schematic flowchart of confirmatory identification of *H. influenzae.*

Identification of the *H. influenzae* serotype

Laboratory identification of *H. influenzae* includes testing for X and V factor requirements and then performing serotyping; this sequence of testing is an efficient way to save costly antisera. However, when the laboratory results must be obtained rapidly for clinical decision-making, serotyping should be performed first the prompt presumptive identification of *H. influenzae.* Isolates identified as *H. influenzae* with typing antisera should still be confirmed by testing for X and V factor requirements.

FIGURE 1: Flowchart for laboratory identification of ***Haemophilus influenzae***



H. influenzae is currently recognized to have six serotypes: a, b, c, d, e, and f. *H. influenzae* type b (Hib) is the major cause of both *H. influenzae* meningitis and of meningitis overall in unvaccinated children in many parts of the world. Suspected Hib isolates should be tested with Hib antiserum, an antiserum to one of the other groups, and saline. **A strongly positive (3+ or 4+) agglutination reaction with type b antiserum and no agglutination with antiserum to the other serotypes and saline is rapid evidence of Hib.**[1]

Antisera should be stored in the refrigerator at 4˚C when not in immediate use. Screening an isolate first with polyvalent antiserum (which contains antisera to all six recognized serotypes) and a saline control is convenient and saves resources (*i.e.*, type-specific antisera).

- **If an isolate is positive in polyvalent antiserum** and negative in the saline control, proceed by testing the isolate with type b antiserum if Hib vaccination is uncommon in the patient's geographic region. If the serotype b reaction is negative, test with the remaining type-specific antisera (*i.e.*, a, c, d, e, and f).
  - If Hib disease is unlikely because of widespread vaccination, the culture should be tested with all the type-specific antisera (*i.e.*, a through f).
- **If an isolate is non-agglutinating in the polyvalent antiserum,** it is either non-typeable or is not *H. influenzae.* Therefore, growth factor requirements must be determined to confirm the identity of the isolate as *H. influenzae* or another species of *Haemophilus.*

Slide agglutination test for serotyping suspected* H. influenzae *isolates

a) Clean a glass slide with alcohol (optional if slides are pre-cleaned). Divide the slide into equal sections (*e.g.*, three 25-mm [1-inch] sections for a 25-mm x 75-mm [1-inch x 3-inch] slide) with a wax pencil or other marker.

b) Collect a small portion of growth from the surface of an overnight culture on chocolate agar (**without bacitracin**), a *Haemophilus* ID plate, or *Haemophilus* test medium (HTM) plate with a sterile inoculating loop. Make a moderately milky suspension of the test culture in a small vial with 250 µl (0.25 ml) of formalinized physiological saline. Vortex the suspension, if possible.
   - If only working with several isolates, another option is to make the suspension directly on the slide in 10 µl of formalinized physiological saline per droplet.

[1] Laboratorians are often tempted to test suspect *H. influenzae* isolates only with type b antiserum since because serotype b (Hib) is vaccine preventable; however, **it is of great importance to screen the isolate with a saline control and at least one other antiserum in addition to type b.** Observing agglutination reactions with several antisera in different portions of the same slide permits comparisons and provides evidence that any agglutination in type b antiserum is not just a mild cross-reaction with a different serotype, providing the laboratorian and clinician with a more informed definition of a 'positive' reaction.

- It is not necessary to make a standard suspension for slide serology; however, it should be noted that a "moderately milky suspension" is roughly comparable to a 6 McFarland turbidity standard.

c) For the agglutination reaction, use a micropipettor or a bacteriologic loop to transfer a drop (5–10 µl) of the cell suspension to the lower portion of two sections of the slide prepared in step a, above. Use enough suspension in the droplet so that it does not dry on the slide before testing with the antisera.

d) Add 5–10 µl of polyvalent antiserum above the drop of suspension in one of the test sections on the slide.[2] In an adjacent section of the slide, use the same method to add a (5–10 µl) drop of saline above the final drop of suspension.

- **The loop used in the antiserum must not touch either the cell suspension or the other antisera being tested; if it does, it must not be placed back into the source bottle of antiserum.** If the source antiserum is contaminated, a new bottle must be used.

e) **Using a separate toothpick (or sterile loop) for each section,** mix the antiserum (and control saline) with the corresponding drop of cell suspension. Avoid contamination across the sections of the slide.

f) **Gently rock the slide with a back and forth motion** for up to 1 minute. Do not use a circular motion while rocking, because it can cause the mixtures to run together and contaminate each other. After one minute of rocking, observe the mixed drops and read the slide agglutination reactions under bright light and over a black background, as shown in Figure 2.

g) **Only strong agglutination reactions (3+ or 4+) are read as positive.** In a strong reaction, all the bacterial cells will clump and the suspension fluid will appear clear (see Figures 11 and 42). When a strain reacts with more than one antiserum, or agglutinates in saline, the result is recorded as non-typeable.

- *If strong agglutination occurs in the polyvalent antiserum:* Using the methods described in steps *a* through *f* (above), continue testing the isolate with type b antiserum and other type-specific antisera to identify the serotype.
- *If agglutination does not occur in the polyvalent antiserum:* The isolate is either non-typeable or not *H. influenzae*. Continue by testing the isolate for X and V growth factor requirements to confirm identification as *H. influenzae*.

---

[2] This laboratory manual suggests using a micropipettor or a loop to transfer antiserum from the bottle to the slide (rather than the dropper provided with the bottle of antiserum) because they conserve costly antiserum resources. (Micropipettors permit the precise measurement of antiserum, and the loop method collects only approximately 5–10 µl of antiserum on average; in contrast, the dropper transfers several times this amount in each drop.) Because only 5–10 µl of antisera are required for agglutination reactions to occur using the methods presented here, **using a micropipettor or a loop to transfer antiserum from the bottle to the slide is more cost-effective.**

- *If agglutination occurs in the saline control:* The isolate is recorded as non-typeable. Continue by testing the isolate for X and V growth factor requirements to confirm identification as *H. influenzae*.

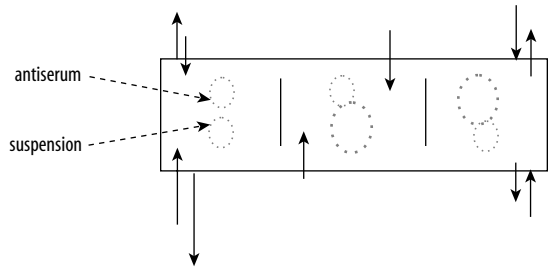
Record results and report to attending clinicians, as appropriate.

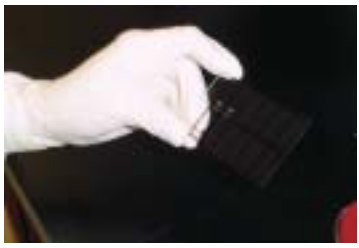
Growth factor (X and V) requirements

H. influenzae is a fastidious organism requiring media containing haemin (X factor) and nicotinamide adenine dinucleotide (NAD, V factor) for growth. The standard medium is chocolate agar, which is often prepared with horse blood, a good source of both X and V factors (Appendix 2). Heating the blood is necessary to make both factors available to the organism. Chocolate agar with added supplements (*e.g.*, IsoVitaleX, Supplement B, or Vitox) is available commercially or can be prepared in the laboratory. Supplemented chocolate agar is superior to unsupplemented medium for growth of *H. influenzae* and is the medium of choice. Although some strains of *H. influenzae* may grow on unsupplemented chocolate agar, **supplements must be added to reliably support the growth of most strains.**

H. influenzae is identified on the basis of its growth requirements for X and V factors (Table 1). *H. influenzae* can be differentiated from most other species of *Haemophilus* by its requirement for both X and V factors for growth.

FIGURE 2: Techniques to properly mix antiserum and suspension for slide agglutination





Gently rock the slide back and forth for slide agglutination reactions.

H. haemolyticus is the only other species requiring X and V factors but this species differs from *H. influenzae* by producing hemolysis on horse- or rabbit blood agar.

Tests to identify X and V growth factor requirements: paper disks and strips or Quad ID plates

Growth factor requirements can be identified with paper disks or strips (using the principles of agar diffusion) or by using Quad ID plates (which contain four types of media with and without X and V factors).

- **Growth factor test using X, V, and XV factor paper disks or strips**
  A medium completely without X and V factors, such as tryptone-based soy agar (TSA) or heart infusion agar (HIA), must be used for this test.

  Methods

  a) Prepare a heavy suspension of cells (1 McFarland turbidity standard, see Appendix 2) from a primary isolation plate in a suitable broth (*e.g.*, tryptone-based soy broth (TSB) or heart infusion broth). If the primary isolation plate contains insufficient growth or is contaminated, make a subculture on a chocolate agar plate. When preparing the broth **avoid transfer of agar medium to the broth;** even the smallest sample of agar will affect the test and **may lead to misidentification** of the bacteria because the agar contains X and V factors.

  b) Inoculate a HIA or TSA plate. A sterile swab or sterile loop of the suspension should be streaked over one-half of the plate (with streaking in at least two directions to ensure confluent growth). Two strains can be tested on one 100-mm plate, but care must be taken to ensure the isolates do not overlap. Paper strips or disks containing X, V, and XV factors are placed on the inoculated plate after the inoculum has dried. **When two bacterial strains are tested on the same plate, as shown in Figure 3, the disks should be placed in the exact manner shown.**

  c) Carefully invert the plate and place it in a CO_2-incubator or candle-extinction jar. Incubate it for 18–24 hours at 35˚C. *H. influenzae* will grow

TABLE 1: Identification of ***Haemophilus*** species by their growth requirements

Species	X- and V-Factor Requirements X	V	ß-hemolysis on rabbit blood agar
H. influenzae	+	+	–
H. parainfluenzae *	–	+	–
H. haemolyticus	+	+	+
H. parahaemolyticus	–	+	+
H. aphrophilus	+	–	–
H. paraphrophilus *	–	+	–

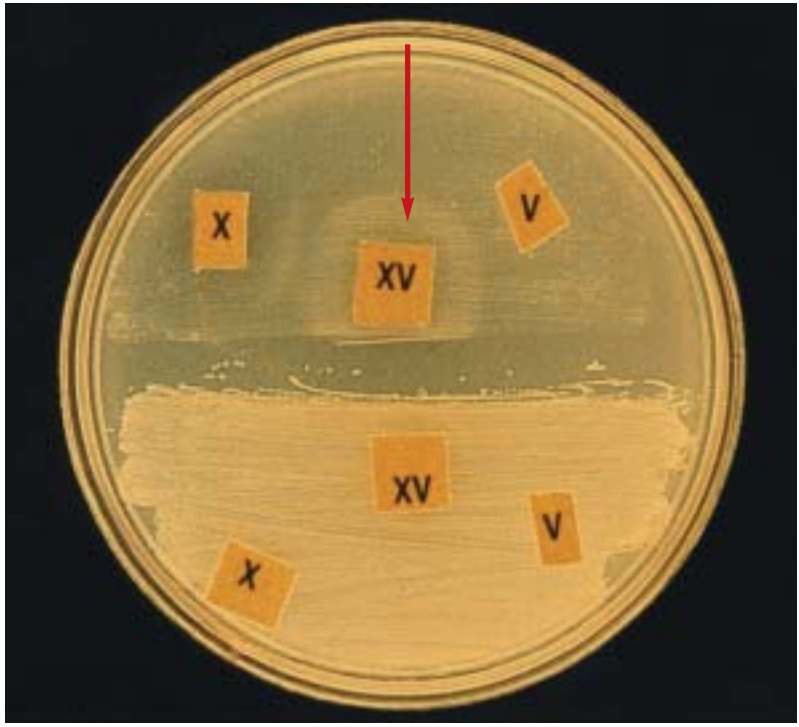
* *H. paraphrophilus* is ornithine negative, whereas *H. parainfluenzae* is ornithine positive.

only around the XV disk (*i.e.*, the disk containing both X and V factors), as shown on the upper half of the plate in Figure 3.

- **Growth factor test using *Haemophilus* Quad ID Plates**

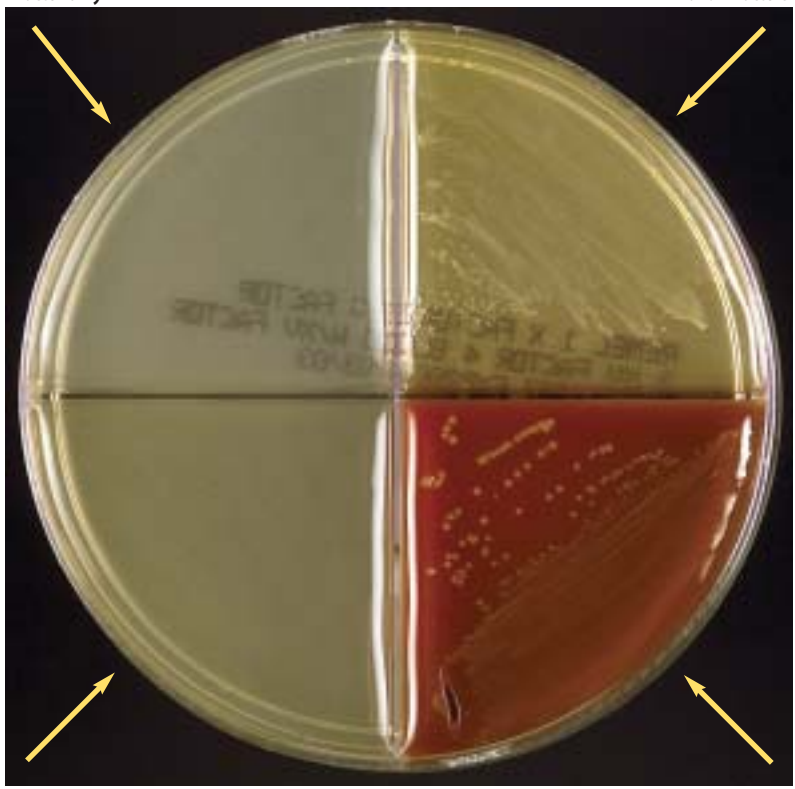
  Quad ID plates are another, although more expensive, method for determining growth factor requirements of *Haemophilus* isolates (Figure 4). Available commercially, the Quad ID plate is divided into four agar quadrants: one quadrant includes medium containing haemin (X factor); one quadrant includes medium containing NAD (V factor); another quadrant contains medium that includes both X and V factors; and, the final quadrant contains heart infusion agar or blood agar base with 5% horse blood for differentiating *H. haemolyticus,* an oral species requiring X and V factors, from *H. influenzae.* Quadrant location of the growth factors may vary with commercial brand of the Quad ID plate.

FIGURE 3: Growth factor requirements: X and V factors on paper disks



The top strain is only growing around the disk containing both X and V factors and can therefore be considered presumptive *H. influenzae.*

FIGURE 4: Growth factor requirements: ***Haemophilus*** Quad ID plate



The Quad ID plate is divided into four compartments. One quadrant (*upper left*) includes medium containing haemin (X factor). One quadrant (*lower left*) includes medium containing nicotinamide dinucleotide (NAD, or V factor). Another quadrant (*upper right*) contains medium that includes both X and V factors. The fourth quadrant (*lower right*) contains heart infusion agar (HIA) with 5% horse blood to detect hemolytic reactions of *Haemophilus* species.

Methods

a) Inoculate the Quad ID plate by suspending the growth from a young, pure culture of suspected *Haemophilus* in tryptone soy broth (TSB) or distilled water to a light, milky suspension (equivalent to a 0.5 McFarland turbidity standard). Using a bacteriological loop, streak one loopful of this suspension on each quadrant of the plate, beginning with the V quadrant and ending with the blood quadrant. Streak the entire quadrant, starting at the periphery and streaking toward the center of the plate. Stab into the blood agar for detection of weak hemolysis.

b) Invert the plate and incubate under a CO_2-enhanced atmosphere (in a candle-jar or CO_2-incubator) for 18–24 hours at 35˚C.

c) After incubation, examine the blood section for hemolysis and the other sections for growth. *H. influenzae* typically shows growth in the XV quadrant and in the (horse-) blood quadrant with no hemolysis. If strong growth occurs in either one of the X or V quadrants besides XV, the organism is probably another species of *Haemophilus.* If growth occurs in every quadrant, the culture is probably not a species of *Haemophilus.* (***Note:*** Occasionally, *H. influenzae* may show slight growth in the V-factor quadrant.) Read and record results.

Hemolytic reactions of *Haemophilus* species

Although most laboratories will not need to determine the hemolytic reaction of each *Haemophilus* spp. (because too few *Haemophilus* strains will be isolated), some laboratories may want to determine the hemolytic reaction to definitively identify both *H. influenzae* and *H. haemolyticus.*

- If X, V, and XV factor disks or strips were used to test growth factor requirements, a separate test to detect hemolytic reactions must be performed by inoculating a broth suspension of the strain on HIA + 5% rabbit blood (or agar infusion base containing horse blood); the hemolytic reaction permits determination the species.
- If a Quad ID plate was used to test for growth factor requirements, the hemolytic reaction of the organism is tested in the (horse-) blood agar quadrant of the plate; thus no separate test is required.

H. influenzae should be α-hemolytic (*i.e.*, causing a greening in the agar around the colony) or γ-hemolytic (non-hemolytic) on the HIA plate containing 5% rabbit blood, while *H. haemolyticus* will exhibit ß-hemolysis (*i.e.*, a clearing of the blood cells in the agar surrounding the colonies on the plate). A summary of test results used in the identification of *H. influenzae* and most closely related *Haemophilus* species is shown in Table 1. Proper determination of the hemolytic reaction is the only way to differentiate *H. influenzae* from *H. haemolyticus.*

Antimicrobial susceptibility testing of *H. influenzae*

The results of antimicrobial susceptibility tests will be used to select the most effective antimicrobial agent to use for treating patients. This laboratory manual describes susceptibility testing of *Haemophilus influenzae* by the disk diffusion method and by the antibiotic gradient strip (Etest®) testing method. Although disk diffusion will provide information as to whether a strain is susceptible,

intermediate, or resistant, the Etest® provides more detailed information about the minimal inhibitory concentration (MIC) of an antimicrobial agent. **The accuracy and reproducibility of these tests are dependent on following a standard set of procedures and conditions in laboratories on an on-going basis.** A sample worksheet for recording antimicrobial susceptibility test results for *H. influenzae* is included in Figure 5.

Media and disks for antimicrobial susceptibility testing

Antimicrobial susceptibility can be determined using the disk diffusion method. The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS;[3] if performed precisely according to the following protocol, this method will provide data that can reliably predict the *in vivo* effectiveness of the drug in question. The accuracy and reproducibility of this test are dependent on the consistent use of a standard set of procedures in laboratories. This section describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results.

The recommended medium for antimicrobial susceptibility testing for *H. influenzae* is *Haemophilus* test medium (HTM) (Appendix 2). The Mueller-Hinton agar used for this test should be thymidine-free to obtain consistent results with trimethoprim-sulfamethoxazole (also referred to as cotrimoxazole). All media used for antimicrobial susceptibility testing should be freshly prepared. Recommended antimicrobial agents for testing are ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole.

The 10-µg ampicillin disk predicts both intrinsic (*i.e.*, penicillin-binding protein-mediated, or "PBP") and ß-lactamase (beta-lactamase) mediated penicillin and ampicillin resistance and should be used when testing *H. influenzae.* (Methods for ß-lactamase testing of *H. influenzae* are listed after the direct antimicrobial susceptibility testing methods in this section.) For *H. influenzae,* a 30-µg chloramphenicol disk is used for predicting resistance to chloramphenicol, and a 1.25/23.75-µg trimethoprim-sulfamethoxazole disk is used for predicting trimethoprim-sulfamethoxazole resistance. **The zone diameter sizes can only be properly interpreted when HTM is used,** as per NCCLS standards.

Quality control of antimicrobial susceptibility testing of *H. influenzae*

Quality control tests must be performed as part of the normal laboratory routine. To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. *H. influenzae* ATCC 49247 is

[3] Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

FIGURE 5: Sample form for recording antimicrobial susceptibility test results for ***Haemophilus influenzae***

Date of Testing: ______/______/_______

*Test performed by:*_________________________

Interpretation of susceptibility: *S* = susceptible *I* = intermediate *R* = resistant

Specimen number	Meningitis isolate?	Organism	Chloramphenicol	Trimethoprim-sulfamethoxazole	Ampicillin	*(other drug)* ________
			mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*
			mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*
			mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*
			mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*
ATCC 49247	N/A	*H. influenzae* NCCLS QC strain *Q/C in range?* √ ¤	mm µg/ml *Yes No*	mm µg/ml *Yes No*	mm µg/ml *Yes No*	mm µg/ml *Yes No*

Reveiwed by: __________ ***Date:*** ____/____/_____

Note: After 16 –18 hours of incubation, check the results for the quality control (QC) strain against the standard acceptable ranges; if they are within control limits, continue reading results for the test isolate. Record disk diffusion results in mm and MIC results in µg/ml. *(Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 2.)*

the control strain used when testing *H. influenzae* for most antimicrobial agents (*e.g.*, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole), although ATCC 49766 is appropriate for some others. (Consult NCCLS document M100-S12 [2002] for more complete information.) **Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits, which are included in Table 2.** If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

- **Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other environmental factors.** The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not.
- **If the depth of the agar in the plate is not uniformly 3–4 mm,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
- **If the pH of the test medium is not between 7.2 and 7.4,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
- **If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected.** For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, even if the isolates are susceptible, when colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks.

Quality control tests should be performed once per week if antimicrobial susceptibility tests are performed daily (after 30 days of in-control results), or with every group of tests when testing is done less frequently. They should also be done with each new batch of test medium and every time a new lot of disks is used.

Antimicrobial susceptibility testing of *H. influenzae* by the disk diffusion method

Prepare the inoculum for seeding the antimicrobial susceptibility media with *H. influenzae* from fresh, pure cultures of *H. influenzae* (*i.e.*, from isolates grown overnight on supplemented chocolate agar). Prepare cell suspensions of the bacteria to be tested in broth or sterile physiological saline; use a suspension equal to a density of a 0.5 McFarland turbidity standard for the inoculum. (Preparation of a McFarland turbidity standard is described in Appendix 2.)

a) Suspend viable colonies from an overnight chocolate agar plate in a tube of broth to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells with the broth. **This suspension should be used within 15 minutes.**

b) Compare the suspension to the 0.5 McFarland turbidity standard by holding the suspension and the McFarland turbidity standard in front of a light against a white background with contrasting black lines and compare the density (see Figures 51 and 52). If the density of the suspension is too heavy, the suspension should be diluted with additional broth. If the density of the suspension is too light, additional bacteria should be added to the suspension.

c) When the proper density is achieved, dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid.

d) Use the swab to inoculate the entire surface of the HTM plate three times, rotating the plate 60 degrees between each inoculation (see Figure 34). Use the same swab with each rotated streak, but **do not re-dip the swab in the inoculum** (*i.e.*, the bacterial cell suspension).

e) Allow the inoculum to dry before the disks are placed on the HTM plates. Drying usually takes only a few minutes, and should take no longer than 15 minutes. (If drying takes longer than 15 minutes, use a smaller volume of inoculum in the future.)

f) After the plate is dry, antimicrobial disks should be placed on the HTM plate as shown in Figure 6. The disks should be placed on the agar with sterile forceps and tapped gently to insure adherence to the agar. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.**

g) Invert the plate and incubate it in a CO_2-enriched atmosphere (5% CO_2-incubator or candle-extinction jar) for 16–18 hours at 35˚C.

- ***Note:*** If this is a new batch of HTM, the antimicrobial disks are new, or it is an otherwise appropriate time to perform quality control, follow steps *a* through *g*- above and run parallel tests on the reference strain(s). Appropriate disk diffusion zone sizes for the reference quality control strain (for the antimicrobial agents included in this chapter) are presented in Table 2.

h) After overnight incubation, measure the diameter of each zone of inhibition. The zones of inhibition on the media containing blood are measured from the top surface of the plate with the top removed. Use either calipers or a ruler with a handle attached for these measurements, holding the ruler over the center of the surface of the disk when measuring the inhibition zone (Figure 6).

- Care should be taken not to touch the disk or surface of the agar. Sterilize the ruler occasionally to prevent transmission of the bacteria. In all

measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony. Record the results in millimeters (mm). Figure 5 provides a sample form for recording results.

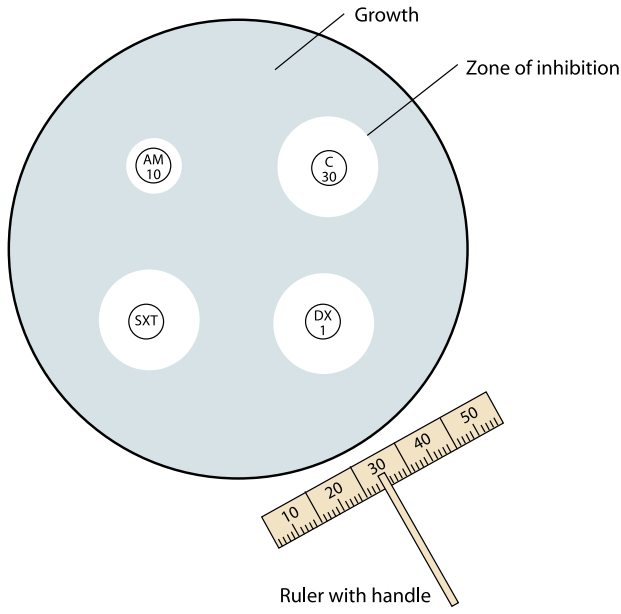
i) Interpretation of the antimicrobial susceptibility is obtained by comparing the results obtained and recorded (in the manner described in this protocol) to the NCCLS standard inhibition zone diameter sizes presented in Table 2.

Minimal inhibitory concentration testing of *H. influenzae* isolates

Laboratorians determining the minimal inhibitory concentration (MIC) for resistant isolates must be highly skilled in performing these tests and committed to obtaining accurate and reproducible results. In addition, a national (or regional) reference laboratory must have the ability and resources to store isolates either by lyophilization or by freezing at -70˚C.

Antimicrobial susceptibility testing by disk diffusion indicates whether an organism is susceptible or resistant to an antimicrobial agent. For surveillance purposes, a laboratory may want to quantify "intermediate" antimicrobial

FIGURE 6: The antimicrobial susceptibility disk diffusion test: disk placement and measurement of inhibition zone diameters



A ruler on a stick can be used to measure zone inibition diameters if calipers are not available.

TABLE 2: Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for ***Haemophilus influenzae***

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml)[a]			NCCLS QC strain ***H. influenzae*** ATCC 49247[b]
		Susceptible	Intermediate	Resistant	
Chloramphenicol	30 µg	> 29 mm *(< 2 µg/ml)*	26– 28 mm *(4 µg/ml)*	< 25 mm *(>8 µg/ml)*	31 – 40 mm *(0.25 – 1 µg/ml)*
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25/ 23.75 µg	≥ 16 mm *(< 0.5/9.5 µg/ml)*	11 mm – 15 mm *(1/18 – 2/36 µg/ml)*	≤ 10 mm *(> 4/76µg/ml)*	24 – 32 mm *(0.03/0.59 – 0.25/4.75 µg/ml)*
Ampicillin	10 µg	≥ 22 mm *(< 1 µg/ml)*	19 mm – 21 mm *(2 µg/ml)*	≤ 18 mm *(> 4 µg/ml)*	13 – 21 mm *(2 – 8 µg/ml)*

[a] *Source:* NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA.

[b] The quality control strain *H. influenzae* ATCC 49247 is appropriate for the testing of the antimicrobial agents included in this table and this laboratory manual overall; however, for testing of some other antimicrobial agents, NCCLS recommends that a different QC strain be used. Laboratories testing the susceptibility of *H. influenzae* to *antimicrobial agents other than those listed* should therefore refer to the NCCLS document M100-S12 (or subsequent updates) for appropriate methods.

susceptibility test results to trimethoprim-sulfamethoxazole detected by disk diffusion testing with MIC testing.

MIC testing by dilution can be expensive and challenging; because of the technical complexity required for these tests, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. In countries where MIC testing is done at more than one laboratory, standardization and quality control should be conducted as described earlier in this chapter.

With increasing antimicrobial resistance testing being performed outside of international reference laboratories, the Etest® serves as a test method that is both convenient and reliable.[4] The Etest® requires less technical expertise than MIC testing by dilution methods, but it gives comparable results. **Etest® strips must be consistently stored in a freezer at -20˚C.**

The Etest® is an antimicrobial susceptibility testing method that is as technically simple to perform as disk diffusion and produces semi-quantitative results that are measured in micrograms per milliliter (µg/ml). It is drug-specific, consists of a thin plastic antibiotic gradient strip that is applied to an inoculated agar plate, and is convenient in that it applies the principles of agar diffusion to perform semi-quantitative testing.[5]

The continuous concentration gradient of stabilized, dried antibiotic is equivalent to 15 $\log_2$ dilutions by a conventional reference MIC procedure as suggested by the

[4] The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

NCCLS. The Etest® has been compared with and evaluated beside both the agar and broth dilution susceptibility testing methods recommended by the NCCLS. Authoritative reports indicate that an (approximately) 85% – 100% correlation exists between the accepted conventional MIC determinations and the MIC determined by the Etest® procedure for a variety of organism-drug combinations (see, *e.g,* Jorgensen *et al.* [1994] and Barry *et al.* [1996] in Appendix 15). Some studies have cited Etest® MICs as approximately one dilution higher than MICs determined by standard dilution methods.

Although this manual serves as a general guide to use of the Etest® antimicrobial gradient strip, **always follow the manufacturer's directions for use of the Etest®,** as certain antibiotic-bacteria ("drug-bug") combinations have special testing requirements.

Methods for antimicrobial susceptibility testing with the Etest®

For *H. influenzae*, HTM is used when performing antimicrobial susceptibility testing. Follow the directions on the package insert included with the Etest® strips. Either 150-mm or 100-mm plates can be used, depending on the number of antimicrobial agents to be tested per isolate. Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and although the manufacturer states that up to six Etest® strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five Etest® strips be used on a 150-mm plate (see Figure 7).

a) Suspend viable colonies from an overnight chocolate agar plate into a broth tube to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells. **This suspension should be used within 15 minutes.**

b) Dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid. Inoculate the entire surface of the agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure confluent growth of the bacteria (see Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.

c) Allow the plate to dry for up to 15 minutes. **Be sure the plate is entirely dry before proceeding.** While the plate is drying, remove the Etest® strips from the -20˚C freezer and allow the strips that will be used in the batch of testing to warm to room temperature. Return the strips that will not be used in this batch of testing to the -20˚C freezer.

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[5] Antimicrobial susceptibility testing with an antimicrobial gradient strip such as the Etest® can be considered to be a semi-quantitative method (because although the suspension used to inoculate a plate for Etest® is standardized, the inoculum itself is not standardized). However, results are generally comparable to quantitative results of standard broth microdilution or agar dilution MIC tests.

d) Place the Etest® strips onto the dried, inoculated agar plate with an Etest® applicator or sterile forceps, oriented as shown in Figure 7. (Make sure that the printed MIC values are facing upward, [*i.e.*, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar].) **Once applied, do not move the antimicrobial gradient strips.**

e) Incubate the plates in an inverted position in a CO_2-enriched atmosphere (2% – 5% CO_2) for 16–18 hours at 35˚C. A candle-extinction jar may be used if a CO_2-incubator is not available.

f) After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **Quality control results must be reviewed before reading and interpreting the Etest® MIC.**

MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read the MIC at the point of complete inhibition of all growth including hazes and isolated colonies. Figure 8 presents a reading guide for the Etest®,[6] and shows drug-related effects, technical and handling effects, organism-related effects and resistance-mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of a *H. influenzae* isolate to ampicillin, an MIC recorded from the graduations on the Etest® strip might be 0.75 mg/ml; however, the reported MIC would be 1.0 µg/ml.

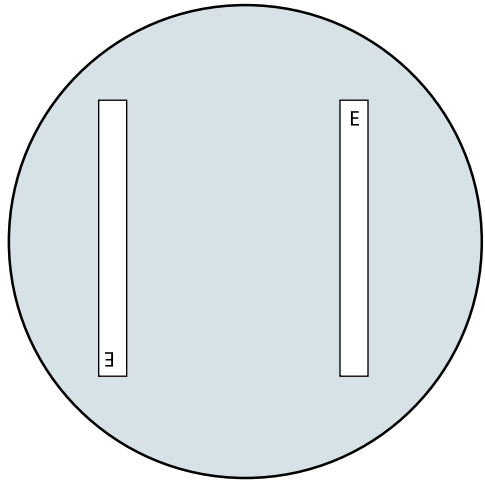
Breakpoints for interpretation of MICs follow the NCCLS guidelines, **unless exceptions made by the manufacturer are provided in the package insert.** NCCLS breakpoints for antimicrobial agents used for *H. influenzae* are included in Table 2.

Surveillance for emerging antimicrobial resistance in *H. influenzae*

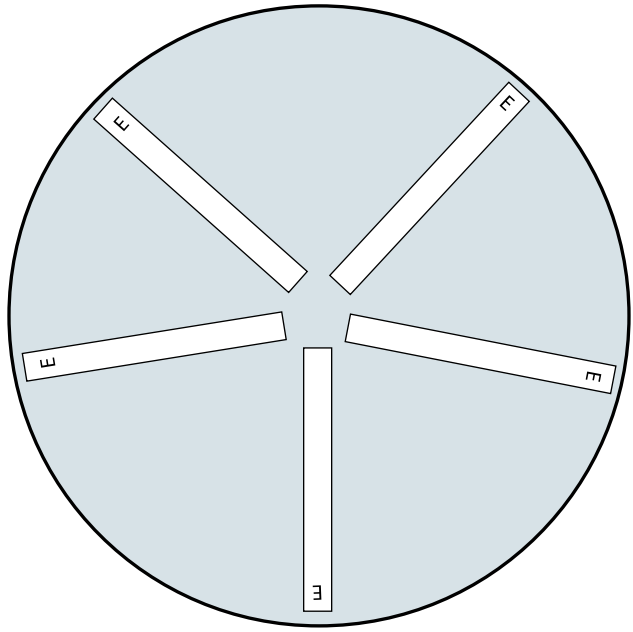
Laboratories may wish to help detect the emergence of new strains of *Haemophilus* by testing isolates against a panel of drugs in which reduced susceptibility is not expected to be found. A laboratory might look at specific drugs or characteristic groupings (such as, for example, ß-lactamase negative, ampicillin resistant

[6] AB Biodisk also maintains a website with an Etest® reading guide: http://www.abbiodisk.com.

FIGURE 7: Proper placement of Etest® strips on dry, inoculated plates



Up to two Etest® strips can be placed on a 100 mm plate, as shown.



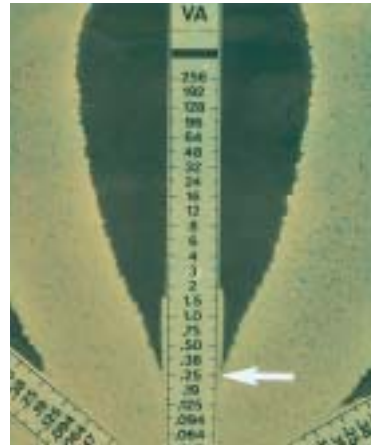
Up to five Etest® strips can be placed on a 150 mm plate, as shown.

FIGURE 8a: Guidance for reading Etest® results

If the strip is backwards,
MIC = INVALID!
Retest and position the strip with the MIC scale facing the opening of the plate.

Intersection in between markings.
Read the next higher value. MIC 0.19 µg/ml.

Different intersections on either side of the strip.
Read the higher value; if the difference is >1 dilution, repeat the test. MIC 0.5 µg/ml.



Ignore a thin line of growth at the edge of the strip caused by organisms growing in a tunnel of water.
MIC 0.25 µg/ml.

The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).

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FIGURE 8b: Guidance for reading Etest® results

Bacteriostatic drugs such as trimethoprim and sulphonamides can give diffuse edges. Read at 80% inhibition. MIC 3 µg/ml.

Isolated resistant colonies due to low-level mutation. MIC >256 µg/ml.

Paradoxical effect showing partial regrowth after an initial inhibition. MIC 8 µg/ml.

Induction of ß-lactamase production by clavulanic acid at the higher MIC range. MIC 96 µg/ml.

The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).

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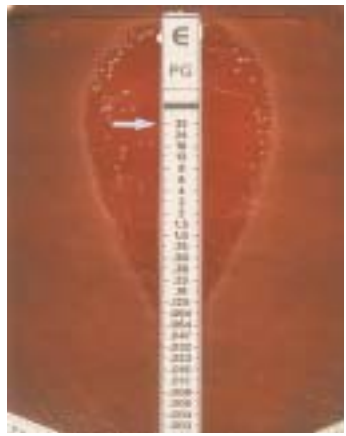
FIGURE 8c: Guidance for reading Etest® results

Tilt the plate to visualize pin-point colonies and hazes. This is particularly important for pneumococci. MIC 1 µg/ml.

Scrutinize pneumococcal end-points carefully to pick up all microcolonies. Tilt the plate and/or use a magnifying glass. MIC 2 µg/ml.



A highly resistant subpopulation in pneumococci. MIC >32 µg/ml.

Encapsulated strains may not give a confluent intersection. MIC 1 µg/ml.

The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).

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[BLNAR] *H. influenzae*). These strains are believed to be rare at present, but are of great interest to public health policy and clinicians because although they may exhibit *in vitro* susceptibility to certain drugs (*e.g.*, amoxicillin + clavulanic acid, cefprozil, cefuroxime, and others), they should still be considered resistant *in vivo* [NCCLS 2002].

Testing for emerging resistance should not be done with each batch of antimicrobial susceptibility tests, nor with each new batch of media. Instead such testing could be done periodically (*e.g.*, on an annual basis), for example on a sampling of preserved isolates in storage on an annual basis. Methods for preservation and long-term storage of isolates can be found in Appendix 11. Antimicrobials of interest could include (but are not necessarily limited to) ceftriaxone and fluoroquinolones. Appropriate zone diameter sizes can be found in NCCLS documents, which are updated regularly. **If any of these rare strains with reduced susceptibility are found in the course of this surveillance, notify an international reference laboratory and submit the isolate for further investigation.** A list of international reference laboratories is included in Appendix 14.

Testing H. influenzae *for ß-lactamase production*

Testing the *H. influenzae* isolates for the presence of ß-lactamase will identify most of ampicillin-resistant strains, because most (but not all) ampicillin resistance among *H. influenzae* is caused by the presence of ß-lactamase. Several techniques are available for the detection of ß-lactamases. All the tests are based on determination of breakdown products and use either a natural substrate (*e.g.*, penicillin) or a chromogenic substance (*e.g.*, nitrocefin). Two methods for detection of ß-lactamase are presented in this manual: the nitrocefin test and the acidometric agar plate method.

- **Nitrocefin** can be used to screen for ß-lactamase either as a reagent dropped onto colonies or in the form of a treated disk onto which colonies are rubbed. (This manual suggests using the disk method unless a laboratory is screening large numbers of isolates because the materials for the reagent tend to be available in bulk and costs can be high; methods for testing with the liquid nitrocefin reagent are included in the *N. gonorrhoeae* chapter [Chapter VI].)
  a) Using sterile forceps or tweezers, place a nitrocefin disk on a clean slide; add a drop of distilled water.
  b) Touch a sterile swab or loop to a characteristic colony from fresh, pure culture.
  c) Rub the swab onto the moistened disk.
  d) Observe the disk for five minutes; if the reaction is positive (ß-lactamase producing strain), the areas of the disk containing growth will turn a characteristic red/pink color.
- A **modified acidometric agar plate method** is a differential agar method for testing *H. influenzae* isolates for the presence of ß-lactamase activity [Park *et al.*

1978; Lucas 1979]. Penicillin and phenol red are combined in a non-nutrient plate; the pH indicator detects increased acidity resulting from the cleavage of the ß-lactam ring of penicillin that yields penicilloic acid, and leads to a color change in the agar.

a) Place a clump of isolated colonies in a discrete spot on the ß-lactamase agar plate. Many strains can be tested on one plate; be certain to note their specific positions with proper labels.
b) Apply known ß-lactamase-positive and ß-lactamase-negative control strains to the plate; label their positions.
c) Incubate the plate in ambient air at 35°C for 15 minutes.
d) Observe the plate for color change in the agar surrounding each discretely spaced colony. The agar surrounding positive-control strain should be yellow, whereas the agar surrounding the negative-control strain should not exhibit any change in color.

Data for decision-making

Once the laboratory has assessed the serotype and antimicrobial susceptibility patterns of *H. influenzae* isolates, the information should be reported back to public health officials promptly. Factors to consider in the development of treatment policy include:

- Childhood immunizations should be considered if *H. influenzae* type b is a major local cause of invasive disease.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and the specific antimicrobial susceptibility profile. National recommendations for empiric antibiotic utilization should be developed after considering antimicrobial susceptibility data, cost, availability, convenience, and other factors.

CHAPTER IV

Neisseria meningitidis

CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Neisseria meningitidis is the etiologic agent of meningococcal disease, most commonly meningococcal bacteremia and meningitis. These two clinically overlapping syndromes may occur simultaneously, but meningitis alone occurs most frequently. *N. meningitidis* is an encapsulated bacterium and is classified into serogroups based on the immunological reactivity of the capsule's polysaccharide. The most common serogroups causing disease are A, B, C, Y, and W135. During the past 20 years, serogroups B and C have been responsible for most meningococcal disease in the Americas and Europe; serogroup A accounts for most meningococcal disease cases in Africa and some parts of Asia.

Meningococcal disease differs from the other leading causes of bacterial meningitis because of its potential to cause large-scale epidemics. Historically, these epidemics have been typically caused by serogroup A and, to a lesser extent, serogroup C. In Africa, the highest incidence rates of serogroup A meningococcal disease occur in a region of Sub-Saharan Africa extending from Sudan in the east to The Gambia in the west; this region consists of 15 countries comprised of more than 260 million people and has been referred to as the "meningitis belt." During epidemics, children and young adults are most commonly affected, with attack rates as high as 1,000/100,000 population, or 100 times the rate of sporadic disease. The highest rates of endemic or sporadic disease occur in children less than 2 years of age. In recent years, two major epidemics of meningitis caused by *N. meningitidis* serogroup W135 have also been reported. In 2000, an outbreak of meningococcal disease in Saudi Arabia (which resulted in 253 cases and 70 deaths) was caused by a virulent clone of serogroup W135; this outbreak occurred simultaneously with the annual pilgrimage to Mecca and returning pilgrims disseminated this clone throughout the world, resulting in secondary cases. As of the time of writing of this laboratory manual in mid-2002, a serogroup W135 meningitis epidemic has been reported in Burkina Faso with more than 12,000 cases and 1400 deaths to date.

A quadrivalent polysaccharide vaccine that includes serogroups A, C, Y, and W135 is produced and used in the United States; however, bivalent A and C polysaccharide vaccines are being used in other parts of the world. New meningococcal conjugate vaccines are under development.

Laboratory personnel at risk for exposure to aerosolized *N. meningitidis* should ensure their protective vaccination status remains current and, if possible, work in a biological safety cabinet. Laboratory scientists who manipulate invasive *N. meningitidis* isolates in a manner that could induce aerosolization or droplet formation (including plating, subculturing, and serogrouping) on an open bench top and in the absence of effective protection from droplets or aerosols should consider antimicrobial chemoprophylaxis.

Confirmatory identification of *N. meningitidis*

The following steps are recommended to confirm the identity of cultures that morphologically appear to be *N. meningitidis* (Figure 9). The best results are obtained with day-old cultures. Always check for purity of the growth by performing a Gram stain: *N. meningitidis* is a gram-negative, kidney-bean- or coffee-bean-shaped diplococcus (see Figure 72). When necessary, make subcultures to ensure purity. From growth on a blood agar plate, perform Kovac's oxidase test, and then identify the serogroup with a slide agglutination test. Finally, confirm the results with carbohydrate (*i.e.*, sugar) reactions.

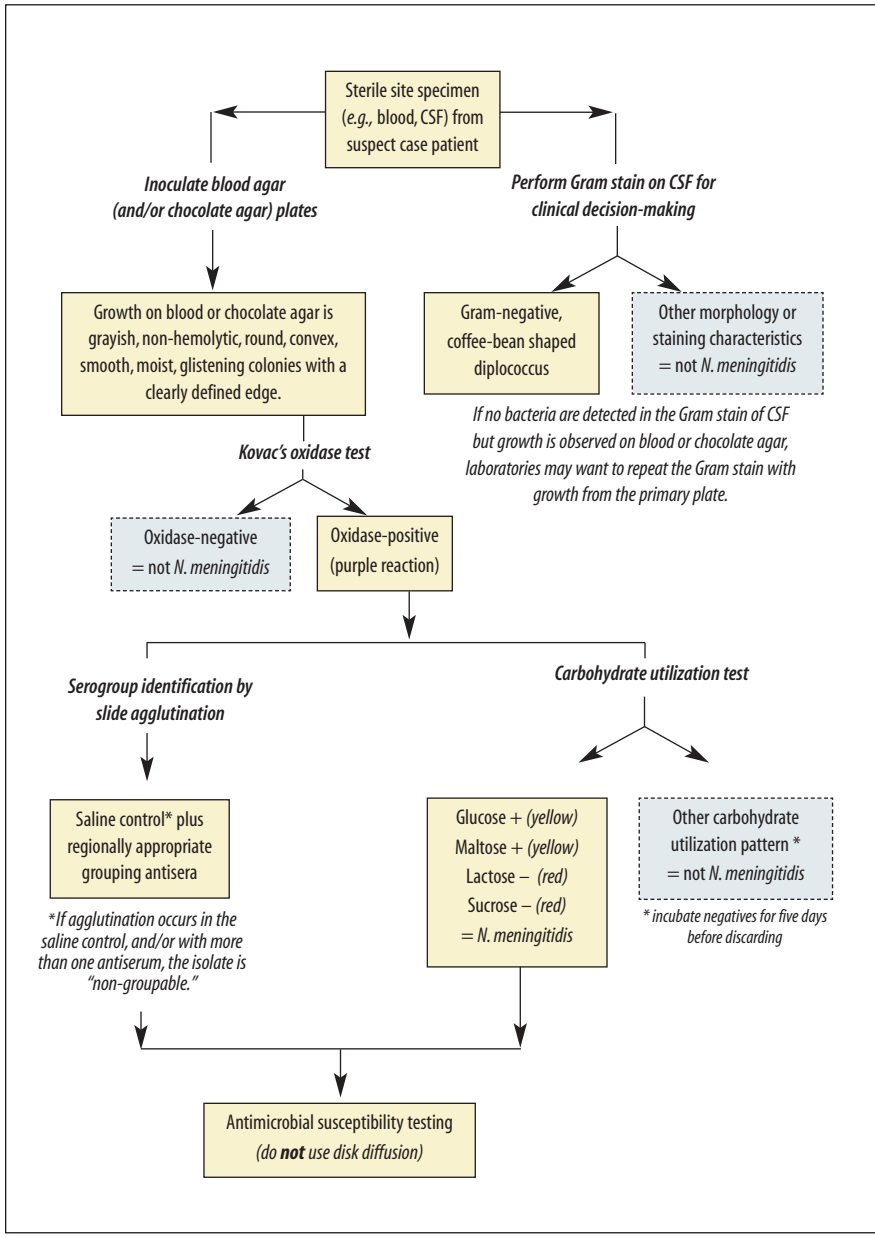
Some laboratorians may be interested in the OMP (*i.e.*, outer membrane protein) subtyping of *N. meningitidis* isolates; these tests may be performed by international reference laboratories.

Kovac's oxidase test for the identification of *N. meningitidis*

The oxidase test determines the presence of cytochrome oxidase. The Kovac's oxidase reagent (1% tetramethyl-ρ-phenylenediamine hydrochloride)[7] is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain; therefore, an oxidase-positive test will yield a purple reaction. (Instructions for making oxidase reagent are found in Appendix 2.)

[7] Some laboratories may use a different reagent, Gordon and MacLeod's reagent, (1% [wt/vol] dimethyl-ρ-phenylenediamene dihydrocholoride; "dimethyl reagent") to perform the oxidase test. The dimethyl reagent is more stable than the tetramethyl reagent (Kovac's reagent), but the reaction with the dimethyl reagent is slower than that with the tetramethyl reagent. **If the laboratory is using the dimethyl- reagent,** a positive reaction will be indicated by a color change to blue on the filter paper (not purple, as with the tetramethyl reagent), and **with the dimethyl reagent it will take 10 – 30 minutes for a positive reaction to develop.**

FIGURE 9: Flowchart for laboratory identification of ***Neisseria meningitidis***



a) Using a platinum inoculating loop, a disposable plastic loop, or a wooden applicator stick, pick a portion of the colony to be tested and rub it onto a treated strip of filter paper (Figure 10). Do not use a Nichrome loop because it may produce a false-positive reaction.

b) Positive reactions will develop within 10 seconds in the form of a purple color. Delayed reactions are unlikely with *N. meningitidis*.

The oxidase test aids in the recognition of *N. meningitidis* and other members of the genus *Neisseria*; other, unrelated, bacterial species with cytochrome c in the respiratory chain (*e.g.*, *Pseudomonas aeruginosa* and *H. influenzae*) are also oxidase positive.

Identification of the *N. meningitidis* serogroup

Twelve serogroups based on capsular polysaccharides are currently recognized: A, B, C, H, I, K, L, W135, X, Y, Z, and Z' (29E). (***Note:*** serogroup D is no longer recognized.) Groups A and C are the common causes of meningitis outbreaks in Africa, but recently outbreaks caused by groups W135 and X have been reported; group B is a cause of endemic meningitis and may also cause outbreaks in some regions of the world (*e.g.*, in Brazil). Grouping antisera are available commercially.

Serogrouping can be expensive, but it is valuable. Serogroup data provides laboratories and public health authorities with the tools to:

- identify outbreaks controllable by a vaccination campaign
- recognize the presence of serogroups causing sporadic disease
- detect the emergence of new outbreak strains (*e.g.*, X or W135).

It is therefore essential that high-level reference laboratories have the capacity to isolate, identify and confirm the serogroup of *N. meningitidis* isolates causing sporadic disease as well as those they receive during the course of an outbreak.

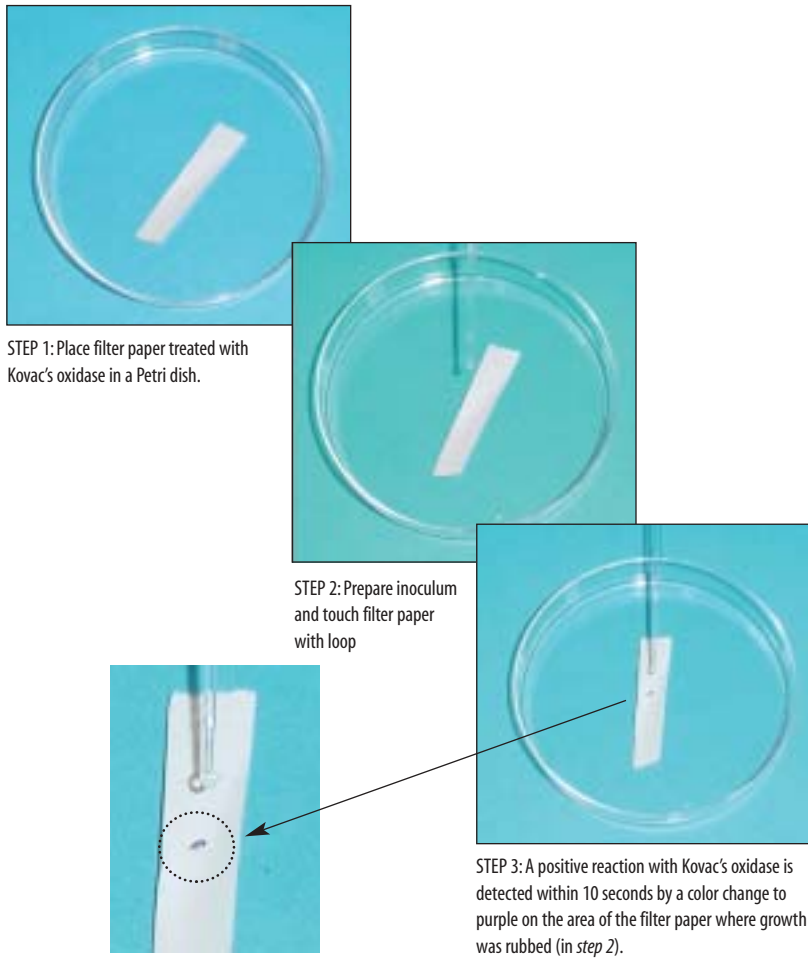
Slide agglutination test for serogrouping suspected N. meningitidis *isolates*

The following methods require both formalinized physiological saline to make the meningococcal suspension and unformalinized physiological saline (or phosphate buffered saline [PBS]) to mix with the antisera. Store antisera in the refrigerator at 4˚C when not in immediate use.

a) Clean a glass slide with alcohol (optional if slides are pre-cleaned). Divide the slides into equal sections (*e.g.*, three 25-mm [1-inch] sections for a 25-mm x 75-mm [1-inch x 3-inch] slide) with a wax pencil or other marker.

b) Collect a small portion of growth from the surface of an overnight culture on non-selective blood or chocolate agar plate using a sterile inoculating loop.

FIGURE 10: Kovac's oxidase test: a positive reaction on filter paper



Make a moderately milky suspension of the test culture in 250 µl (0.25 ml) of formalinized physiological saline. Vortex the suspension, if possible. If working with only several isolates, it may be more convenient to make the suspension directly on the slide in 10 µl of formalinized physiological saline per droplet.

- ***Note:*** For safety reasons, it is recommended that formalin-killed meningococcal suspensions rather than saline suspensions of living organisms be used; however, formalin is a carcinogen and must be stored

and handled with great care. (Alternatively, if formalin is not used to kill the meningococci, laboratorians can work under a safety hood.)

- It is not necessary to make a standard suspension for slide serology; however, it should be noted that a "moderately milky suspension" is roughly comparable to a 6 McFarland turbidity standard.

c) Use a micropipettor or a bacteriologic loop to transfer a drop (5–10 µl) of the cell suspension to the lower portion of each section of the slide prepared in step *a* of this procedure.[8]

d) Add a drop of group A antiserum above the drop of suspension in one of the test sections on the slide. In one of the other sections of the slide, add a drop of W135 antiserum below the drop of suspension in that section. For the third section of the slide, use the same method to add a drop of saline below the final drop of suspension.

- **The loop used in the antiserum must not touch either the cell suspension or the other antisera being tested; if it does, it must not be placed back into the source bottle of antiserum.** If the source antiserum is contaminated, a new bottle must be used.
- ***Note:*** In Africa, testing with A and W135 antisera (with a saline control to detect nonspecific autoagglutination) should be adequate for serologic characterization of most *N. meningitidis* isolates. Strains reacting negatively with A and W135 antisera should then be tested with other available antisera, particularly C, Y, B, and X.

e) **Using a separate toothpick (or sterile loop) for each section,** mix each antiserum (and control saline) with its corresponding drop of cell suspension. Avoid contamination across the sections of the slide.

f) **Gently rock the slide with a back and forth motion** for up to 1 minute. Do not use a circular motion while rocking, as it can cause the mixtures to run together and contaminate each other. After one minute of rocking, observe the three mixed drops and read the slide agglutination reactions under bright light and over a black background (see Figure 2).

g) **Only strong agglutination reactions (3+ or 4+) are read as positive.** In a strong reaction, all the bacterial cells will clump and the suspension fluid will appear clear (see Figure 11 and Figure 42). When a strain reacts only in one grouping antiserum, it should be recorded as belonging to that serogroup.

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[8] This laboratory manual suggests using a micropipettor or a loop to transfer antiserum from the bottle to the slide (rather than the dropper provided with the bottle of antiserum) because they conserve costly antiserum resources. (Micropipettors permit the precise measurement of antiserum, and the loop method collects only 5–10µl of antiserum on average; in contrast, the dropper transfers several times this amount in each drop.) Because only 5–10 µl of antisera are required for agglutination reactions to occur using the methods presented in this manual, **using a micropipettor or a loop to transfer antiserum from the bottle to the slide is more cost-effective.**

(For example, an isolate exhibiting a strong agglutination reaction only in group A antiserum would be recorded as '*N. meningitidis*, serogroup A.')

- *If a strong agglutination reaction does not occur with the antisera tested:*
  - If the isolate is negative in the first two antisera tested (groups A and W135 in Africa) and the saline control, repeat the test with different antisera to identify the serogroup, following steps a through f of this procedure.
- *When a strain reacts with more than one antiserum or agglutinates in saline, the strain is categorized as non-groupable.* (These results occur rarely with fresh isolates, but they do happen occasionally.) Non-groupable results are characterized by:
  1) Autoagglutination in the saline control ("autoagglutinable").
  2) Cross-agglutination with reactions in more than one antiserum ("rough").
  3) No agglutination with either any of the antisera or with the saline control ("non-reactive").

Report results of *N. meningitidis* serogroup testing back to attending clinicians, as appropriate.

FIGURE 11: Positive and negative agglutination reactions on a slide: grouping antisera and saline control with ***Neisseria meningitidis***

When a suspension is mixed with its homologous antiserum, agglutination occurs (*left*). In a negative reaction, as shown with a heterologous antiserum (*center*) or control saline (*right*), the suspension remains smooth and cloudy in appearance.

Carbohydrate utilization by *N. meningitidis*: cystine trypticase agar method

Carbohydrate utilization tests are used to further validate the identification of a strain as *N. meningitidis*. Various carbohydrates are added to the cystine trypticase agar (CTA) base to a final concentration of 1%. To confirm a culture as *N. meningitidis*, a set of four tubes, each containing a sugar (*i.e.*, glucose [dextrose], maltose, lactose, and sucrose) is used. Members of *Neisseria* species produce acid from carbohydrates by oxidation, not fermentation. *N. meningitidis* oxidizes glucose and maltose, but not lactose and sucrose. A phenol red indicator is included in the medium; it is a sensitive indicator that develops a yellow color in the presence of acid, at a pH of 6.8 or less. (Methods for the preparation and quality control of CTA medium are included in Appendix 2.)

a) With an inoculating needle, collect a small amount of growth from an overnight culture of *N. meningitidis* on blood agar or chocolate agar.

b) Stab the inoculum several times into the upper 10 mm of medium. Use another sterile needle, or flame the same needle, before inoculating each of the four carbohydrates to be tested.

c) Fasten caps of tubes **tightly** and place in a 35°C incubator (without CO_2). Incubate for at least 72 hours (and up to 5 days) before discarding as negative.

d) Development of visible turbidity and a yellow color in the upper portion of the medium indicates growth and the production of acid and is interpreted as a positive test (Figure 12). Although reactions may occur as early as 24 hours after inoculation, some reactions are delayed. If only glucose or maltose or none of the sugars react, continue incubation for up to 5 days before discarding. Occasionally, strains of *N. meningitidis* are encountered that utilize only dextrose or maltose but not both (Table 3).

TABLE 3: Carbohydrate utilization by some species of ***Neisseria*** and ***Moraxella***

	Produces acid from [a]			
Species	Glucose [b]	Maltose	Lactose	Sucrose
N. meningitidis	+	+	–	–
N. gonorrhoeae	(+) [c]	–	–	–
N. sicca	+	+	–	+
N. lactamica	+	+	+	–
M. catarrhalis	–	–	–	–

[a] Negative results should not be interpreted prior to 72 hours of incubation in order to avoid false-negative results for delayed acid production reactions.

[b] Glucose may be also be referred to as "dextrose".

[c] Strains of *N. gonorrhoeae* that are weak acid producers may appear to be glucose-negative in cystine trypticase agar (CTA) medium.

FIGURE 12: Cystine trypticase agar sugar reactions for acid production from carbohydrates by ***Neisseria meningitidis***

Acid is produced by utilization of sugar and causes the CTA medium to turn yellow at or just below the agar surface. For *N. meningitidis,* there is utilization of dextrose and maltose (*two tubes on left with yellow color just below the surface*) and no utilization of lactose nor sucrose (*two tubes on right with solid red color of medium*).

Commercial identification kits for *Neisseria*

Several commercial identification systems that use biochemical or enzymatic substrates are available for identification of *Neisseria* species. These systems may occasionally require supplemental tests, and other characteristics, such as microscopic and colony morphology, must be considered; additionally, antimicrobial susceptibility testing cannot be conducted without a confirmed *N. meningitidis* isolate. Generally, each system is self-contained, but addition of one or more reagents to complete certain reactions may be necessary. Follow the manufacturer's instructions precisely when using these kits. For detailed instructions and use of appropriate control strains, also consult the *Clinical Microbiology Procedures Handbook* (see Appendix 15). Rapid sugar utilization test kits may also be used toward the identification of *N. meningitidis.*

Antimicrobial susceptibility testing of *N. meningitidis*

N. meningitidis does not commonly show resistance to many antimicrobial agents. Low-level resistance to penicillin is common in some areas of the world, though the clinical significance of this resistance has not yet been established. Meningococcal resistance to sulfonamides, rifampicin (also referred to as rifampin), and chloramphenicol has also been described. Chloramphenicol tends to be the empiric drug of choice for treating patients with meningitis caused by *N. meningitidis*; rifampicin and sulfonamides are often used for prophylaxis.

Antimicrobial susceptibility testing of *N. meningitidis* should not be performed by disk diffusion, even though it is the least expensive screen, because results are very difficult to interpret and will not provide data useful for making informed treatment decisions. Two appropriate methods for testing include (1) minimal inhibitory concentration (MIC) determination by broth microdilution, and (2) use of the Etest® strip. The broth microdilution methodology provides laboratorians with quantitative MIC results based on the inhibition of growth of a standard inoculum in standard concentrations (dilutions) of antimicrobial. The Etest® antimicrobial susceptibility test methodology provides laboratorians with semi-quantitative MIC results, because although a standard suspension is used to inoculate a plate, the inoculum is not precisely standardized. Results of the Etest® and conventional MIC testing by broth microdilution are generally comparable.

The broth microdilution procedure can be expensive and challenging to perform and, because of the technical complexity required, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. For laboratories that do not perform MIC testing by dilution methods but still want to perform antimicrobial susceptibility tests on *N. meningitidis* isolates, the Etest® may be a convenient alternative.[9] The Etest® is easier to quality control and is the focus of this section of the manual, but broth microdilution methodology is included in Appendix 7. Figure 13 shows a sample worksheet for recording antimicrobial susceptibility test results for *N. meningitidis*.

Either 150-mm or 100-mm plates can be used for the Etest®, depending on the number of antimicrobial agents to be tested per isolate. Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and although the manufacturer states that up to six Etest® strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five Etest® strips be used on a 150-mm plate (see Figure 7).

[9] The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

FIGURE 13: Sample form for recording antimicrobial susceptibility test results for ***Neisseria meningitidis***

Date of Testing: ____/____/_____
*Test performed by:*_____

Interpretation of susceptibility: *S* = susceptible *I* = intermediate *R* = resistant

Specimen number	Organism	Penicillin	Rifampicin *(Rifampin)*	Trimethoprim-sulfamethoxazole	Chloramphenicol	*(other drug)* _____
		µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***
		µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***
		µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***	µg/ml ***S I R***
S. pneumoniae ATCC 49619 [a]	QC strain *QC in range?* →	µg/ml *Yes No*	µg/ml *Yes No*	µg/ml *Yes No*	µg/ml *Yes No*	µg/ml *Yes No*

[a] NCCLS has not validated MICs for *N. meningitidis*; this laboratory manual suggests using *S. pneumoniae* ATCC 49619 as the control strain.
[Source: Dr. F. Tenover, Centers for Disease Control and Prevention, Atlanta, GA USA: 2002.]

Reveiwed by: _____ ***Date of Report:*** ____/____/_____

Note: After 18 – 22 hours of incubation, check the results for the quality control (QC) strain against the standard acceptable ranges; if they are in control, continue reading results for the test isolate. Record MIC results in µg/ml. *(Breakpoints for interpretation of results may be found in Table 4.)*

Minimal inhibitory concentration testing of *N. meningitidis* by Etest® antimicrobial gradient strip

Mueller-Hinton + 5% sheep blood agar is used when testing *N. meningitidis* isolates with the Etest®. Follow the directions on the package insert included with the Etest® strips.

a) Using a sterile cotton-tip applicator, touch the surface of one to four morphologically similar, isolated colonies grown on a chocolate agar plate incubated in CO_2-enhanced atmosphere (5% CO_2 in a CO_2-incubator or candle-extinction jar) at 35˚C for 18–22 hours. Immerse the applicator into a tube containing sterile broth (*e.g.*, Muller-Hinton broth). Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix the cells to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. **This suspension should be used within 15 minutes.**

b) Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard. If the turbidity of the inoculum is greater than the standard, dilute it with broth to equal the turbidity of the standard. (Figures 51 and 52 in Appendix 2 show how to compare the turbidity of the suspension with the standard and also provide black and white lines as a reading background.)

c) Immerse a sterile cotton-tipped swab into the adjusted inoculum (prepared in step *b* of this procedure). Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a 15x150-mm Mueller-Hinton + 5% sheep blood agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure even distribution of the inoculum and confluent growth of the bacteria (see Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.

d) Allow the inoculum to dry on the surface of the plate (which should take approximately 10 minutes). **Be sure the plate is entirely dry before proceeding.** While the plate is drying, remove the Etest® strips from the -20˚C freezer, and allow the strips that will be used in the batch of testing to warm to room temperature. Return the antimicrobial gradient strips that will not be used in this batch of testing to the -20˚C freezer.

e) When the surface of the inoculated plate is dry and the Etest® strips are at room temperature, place the antimicrobial gradient strips onto the agar with an Etest® applicator or sterile forceps, as illustrated in Figure 7. Make sure that the printed MIC values are facing upward (*i.e.*, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar.) **Once applied, it is important to not move the antimicrobial gradient strips.**

f) Incubate the plates in an inverted position in a 5% CO_2 atmosphere for 18–22 hours at 35˚C. A candle-extinction jar may be used if a CO_2-incubator is not available. Because *N. meningitidis* grows well in a humid atmosphere, laboratorians may choose to add a shallow pan of water to the bottom of the incubator or add a dampened paper towel to the candle-extinction jar.

After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **Quality control results must be reviewed before reading and interpreting the Etest® MIC.** MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read at the point of complete inhibition of all growth including hazes and isolated colonies. Figure 8 presents a reading guide for the Etest®,[10] and shows drug-related effects, technical and handling effects, organism-related effects, and resistance mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of an isolate to penicillin, an MIC recorded from the graduations on the Etest® strip might be 0.094 μg/ml; however, the reported MIC would be 0.125μg/ml.

***Quality control for antimicrobial susceptibility testing of* N. meningitidis**

To verify that antimicrobial susceptibility test results are accurate, it is important to include at least one control organism. It is of note here that NCCLS[11] does not publish MIC ranges specific to *N. meningitidis*; however, the Centers for Disease Control and Prevention (CDC, United States of America) recommends that if antimicrobial susceptibility testing of *N. meningitidis* is going to be performed, then a banked control strain for fastidious organisms (*S. pneumoniae* ATCC 49619) should be used for quality control. The NCCLS MIC ranges for quality control testing of *S. pneumoniae* ATCC 49619 with the antimicrobial agents penicillin, rifampicin, and sulfonamides are included in Table 4. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

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[10] AB Biodisk also maintains a website with an Etest® reading guide: http://www.abbiodisk.com.

[11] Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

TABLE 4: Minimal inhibitory concentration (MIC) ranges for quality control of ***Neisseria meningitidis*** antimicrobial susceptibility testing

QC strain for ***N. meningitidis*** [a]	Penicillin MIC Range [b]	Rifampicin (Rifampin) MIC Range [b]	Trimethoprim-sulfamethoxazole MIC Range [b]	Chloramphenicol MIC Range [b]
S. pneumoniae ATCC 49619	0.25 – 1 µg/ml	0.015 – 0.06 µg/ml	0.12/2.4 – 1/19 µg/ml	2 – 8 µg/ml

[a] *Source:* Dr. F. Tenover, Centers for Disease Control and Prevention, Atlanta, Georgia, USA: 2002.
[b] *Source:* NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.

Resistance to antimicrobials other than penicillins and rifampicin is not commonly detected in *N. meningitidis*; however, laboratories, clinicians, and other public health practitioners may be interested in performing annual screens of isolates in storage. (Appendix 11 provides methods for how to preserve and store meningococcal isolates.) Periodic, non-routine surveillance for characteristics such as ß-lactamase production, and ceftriaxone, chloramphenicol and fluoroquinolone resistance will help provide information to public health agencies and international reference laboratories regarding the emergence of new *N. meningitidis* strains of clinical and public health concern.

Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct for trimethoprim-sulfamethoxazole (cotrimoxazole); organisms may then appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm or the pH is not between 7.2 and 7.4, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected. (**Do not attempt to adjust the pH of this Mueller-Hinton agar** even if it is out of range; see Appendix 2)

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected. For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks even when the isolates being tested are susceptible.

Reading and interpreting the Etests®

Read the MIC at the point where the zone of inhibition intersects the MIC scale on the strip, as illustrated in Figure 8. Record the quality control results first. If zones

produced by the control strain are out of the expected ranges (Table 4), the laboratorian should consider possible sources of error. If all antimicrobial agents are in control, read the test MICs. Note any trailing endpoints.

Because antimicrobial susceptibility test results can be affected by many factors not necessarily associated with the actual susceptibility of the organism (*e.g.*, inoculum size, agar depth, storage, time, and others), quality control practices must be followed carefully.

Although NCCLS has not defined standardized breakpoints for the interpretation of an *N. meningitidis* isolate as susceptible or not, the MICs obtained by antimicrobial susceptibility testing methods as described in this document can still be used. Just as a laboratory might assess antimicrobial susceptibility for the very many other organisms for which no NCCLS breakpoints have been defined, laboratorians and clinicians should consider the site of infection in conjunction with the dose and pharmacokinetics of the antimicrobial agent to determine how much drug reaches the site of the infection. This information should then compared to the MIC value to determine if the concentration of drug available is at least four times greater than the MIC. If the concentration of drug available is ≥4 times the MIC, the organism may be considered susceptible; if not, it is resistant.

Data for decision-making

Once the laboratory has confirmed the identification and serogroup (and antimicrobial susceptibility patterns, if appropriate) of *N. meningitidis* isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- Immunization should be considered if a *N. meningitidis* vaccine serotype is a major cause of local invasive disease.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and the specific antimicrobial susceptibility profile. National recommendations for empiric antibiotic utilization should be developed after considering antimicrobial susceptibility data, cost, availability, convenience, and other factors.

CHAPTER V

Streptococcus pneumoniae

CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Streptococcus pneumoniae is a common agent of lower and upper respiratory diseases, such as pneumonia, meningitis and acute otitis media (middle ear infections), affecting children and adults worldwide. This bacterial pathogen is the cause of approximately 40% of acute otitis media. Although acute otitis media and other upper respiratory tract infections do not commonly progress to invasive disease they do contribute significantly to the burden and cost of pneumococcal disease. Meningitis in infants, young children and the elderly is often caused by *S. pneumoniae*. Persons who have sickle cell disease, anatomic asplenia, or are immunocompromised also have increased susceptibility to *S. pneumoniae* infection. Pneumococcal meningitis is the most severe presentation of disease, but most illnesses and deaths result from pneumococcal pneumonia. Pneumococcal polysaccharide vaccine has been available for preventing invasive disease in the elderly and in persons with chronic illnesses that may impair their natural immunity to pneumococcal disease; however, this vaccine is not effective in children <2 years of age. In contrast to polysaccharide vaccines, conjugate vaccines are effective in young children. A pneumococcal conjugate vaccine covering seven serotypes that most commonly cause bacteremia in children in the United States (and some other industrialized nations) was approved for clinical use in 2000; research on vaccine formulations containing serotypes more common in developing countries is underway.

S. pneumoniae is frequently carried in the throat without causing disease. On occasion, public health investigations call for studies on the prevalence of *S. pneumoniae* carriage. For this research, samples may be collected using nasopharyngeal (NP) swabs; methodology for collection and isolation with NP swabs is included in Appendix 5. Antimicrobial susceptibility testing on isolates should be performed as presented in this chapter.

Confirmatory identification of *S. pneumoniae*

S. pneumoniae are gram-positive diplococci or chains of cocci (see Figure 73). On blood agar and chocolate agar plates, *S. pneumoniae* colonies appear small, greyish and mucoid (*i.e.*, watery), and are surrounded by a greenish zone of alpha-hemolysis (α-hemolysis).

Colonies of pneumococci and α-hemolytic viridans streptococci each appear raised when young; however, after 24–48 hours, the center of pneumococcal colonies becomes depressed, whereas viridans streptococcal colonies retain their raised appearance (Figure 14). A 3x hand-lens or a microscope (30x–50x) can therefore be a useful aid in differentiating pneumococci from α-hemolytic viridans streptococci. Laboratory differentiation between *S. pneumoniae* and viridans streptococci is accomplished by optochin and bile solubility testing: pneumococci are susceptible to optochin and bile-soluble, while viridans streptococci are not. Commercially available slide agglutination tests can also be used for identification of pneumococci. For optimal results plates for pneumococcal identification assays should be incubated in a 5% CO_2 atmosphere.

A flowchart diagram of the laboratory identification of *S. pneumoniae* is included in Figure 15. Presumptive identification of *S. pneumoniae* is made by determining the susceptibility of the strain to optochin (*i.e.*, ethylhydrocupreine). The bile solubility test is also used for identification of *S. pneumoniae*, particularly when results of the optochin susceptibility test are ambiguous.

Optochin susceptibility test

The optochin susceptibility test is performed with a 6-mm, 5-µg optochin disk,[12] and is used to differentiate between *S. pneumoniae* and viridans streptococci. Optochin-susceptible strains can be identified as *S. pneumoniae.*

Performance of the optochin susceptibility test

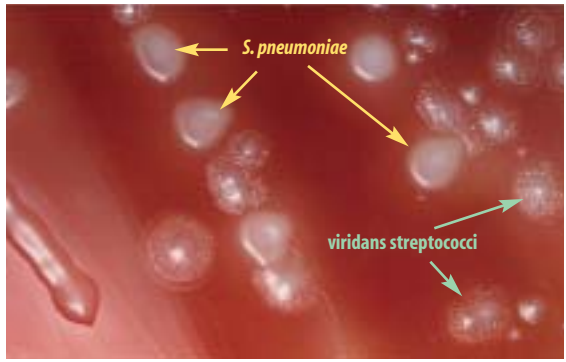
a) Touch the suspect α-hemolytic colony with a sterile bacteriological loop and streak for isolation onto a blood agar plate in a straight line. Several strains can be tested on the same plate at once, streaked in parallel lines and properly labeled.

b) Aseptically place an optochin or "P" disk with a diameter of 6 mm (and containing 5 µg of ethylhydrocupreine) on the streak of inoculum, near the end where the wire loop was first placed. Because the inoculum is streaked in a straight line, three to four colonies may be tested on the same plate (Figure 16).

[12] **The results and interpretation of the optochin susceptibility test presented in this document are appropriate for the 6-mm, 5-µg optochin disk ("P"-disk),** although-different size disks (and possibly optochin concentrations) are available for purchase. When using optochin disks with different size and/or concentration parameters, follow the manufacturer's instructions for interpretation.

FIGURE 14: A properly streaked blood agar plate with pneumococci and viridans streptococci

Note how growth is heavy where streaking began on the left and then thins to individual colonies.



The *S. pneumoniae* has a depressed center (yellow arrows) at 24 – 48 hours incubation, whereas the viridans streptococci retain a raised center (black arrows).

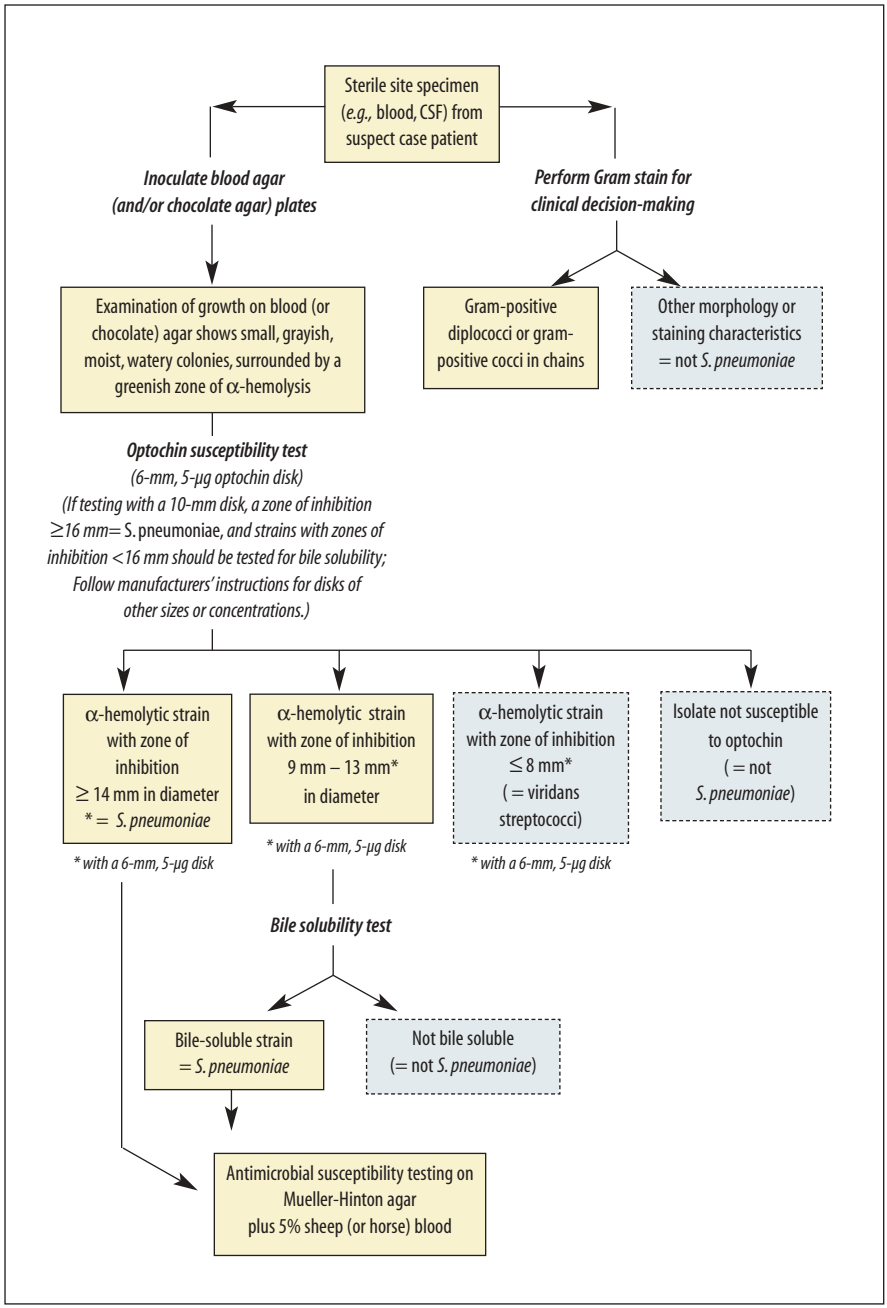
c) Incubate the plates in a CO_2-incubator or candle-jar at 35˚C for 18–24 hours.

d) Read, record, and interpret the results.

Reading and interpreting the optochin susceptibility test results

In Figure 16, the strain in the top streak is resistant to optochin and, therefore, is not a pneumococcus. The strains in the center and lower streaks are susceptible to optochin and appear to be pneumococci.

FIGURE 15: Flowchart for laboratory identification of ***Streptococcus pneumoniae***



- α-hemolytic strains with a zone of inhibition of growth greater than 14 mm in diameter are pneumococci.

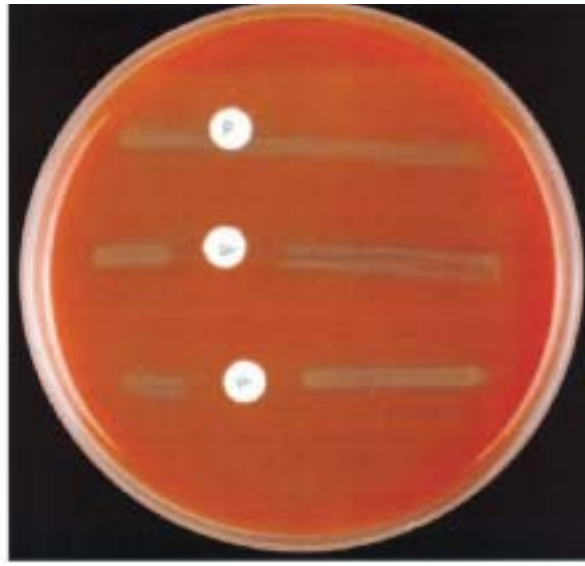
  (If using a 10-mm, 5-µg disk, α-hemolytic isolates with a zone of inhibition of growth >16 mm in diameter are considered susceptible to optochin and, therefore, are pneumococci.)

- α-hemolytic strains with no zones of inhibition are viridans streptococci.

- **α-hemolytic strains with zones of inhibition ranging between 9 mm and 13 mm should be tested for bile solubility** for further characterization and identification.

  (If using a 10-mm disk, α-hemolytic isolates with a zone of inhibition of growth <16 mm should be tested for bile solubility.)

FIGURE 16: Optochin susceptibility test for identification of ***Streptococcus pneumoniae***



The optochin susceptibility test for *S. pneumoniae* uses P-disks (optochin disks); this laboratory manual presents guidelines for interpretation of the optochin susceptibility test based on a 6-mm, 5-µg optochin disk. The strain in the top streak grew up to the disk: it is resistant to optochin and therefore is not a pneumococcus. The strains in the center and lower streaks are susceptible to optochin and appear to be pneumococci.

Bile solubility test

The bile solubility test is performed on isolates with small zones of inhibition in the optochin susceptibility test. It can be performed using either the "tube method" or the "plate method."

Tube method for the performance of the bile solubility test

Two tubes are required for bile solubility testing of each suspect strain of *S. pneumoniae*.

a) Take a loop of the suspect strain from fresh growth on a blood agar plate and prepare a bacterial cell suspension in 0.5 ml of sterile saline. The suspension of bacterial cells should be cloudy, similar to that of a 0.5 or 1.0 McFarland turbidity standard. (Preparation of a McFarland turbidity standard is described in Appendix 2.)
   - If growth on the optochin test plate is sufficient, the suspension can be made with the bacterial cells collected from the specific streak of suspect *S. pneumoniae*.
   - When there is insufficient growth to make a suspension of the proper density in 0.5 ml of sterile saline, inoculate a blood agar plate with the suspect growth and incubate overnight (*i.e.*, for 18 – 24 hours at 35˚C in a CO_2-enriched atmosphere) to prepare a fresh culture.

b) Divide the suspension into two equal amounts (*i.e.*, 0.25 ml per tube). Add 0.25 ml of saline to one tube and 0.25 ml of 2% sodium desoxycholate (bile salts) to the other.
   - To make a 2% concentration of bile salts, add 0.2 g of sodium desoxycholate to 10 ml of saline.

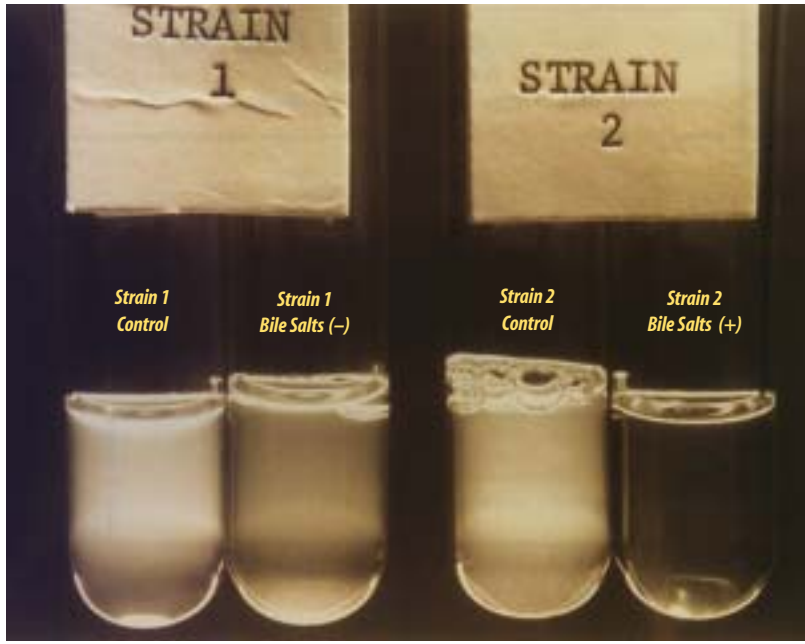
c) Shake the tubes gently and incubate them at 35˚– 37˚C for up to 2 hours.

d) Examine the tubes periodically for lysis of cells in the tube containing the bile salts. A clearing of the tube, or a loss in turbidity, is a positive result (Figure 17).
   - Strains that yield clearing of the suspension in tube in the bile solubility test should be reported as "bile soluble."
   - Strains for which the turbidity in the tube remains the same as that in the saline control tube is reported as negative for bile solubility (or "bile insoluble" or "bile resistant").

Plate method for the performance of the bile solubility test

In place of the tube test for bile insolubility, a laboratorian may perform the bile solubility test using the plate method. **A freshly prepared culture** of the suspect organism must be used for this test.

FIGURE 17: Positive and negative results of the bile solubility test



Results of the bile solubility test are shown for two different strains of bacteria. A slight decrease in turbidity is observed in the tube containing bile salts for strain 1 (*2nd tube from left*), but the contents are almost as turbid as the control tube (*far left*); therefore, strain 1 is not *Streptococcus pneumoniae*. Strain 2 is *S. pneumoniae:* all turbidity in the tube on the far right has disappeared, the clearness indicating that the cells have lysed; in contrast, the control tube (*2nd from right*) is still turbid and cells are intact.

a) Place a drop of 10% sodium desoxycholate solution directly on a colony of the suspect pneumococcal strain to be tested.
   - (To prepare the 10% solution of bile salts, add 1 g of sodium desoxycholate bile salts to 10 ml of sterile saline.)

b) Keep the plate at room temperature (*i.e.*, 72˚–75˚C) or place it face up (*i.e.*, agar-side up) and on a level surface in an ambient air incubator (*i.e.*, **not** a CO_2-incubator) at 35˚C for approximately 15 minutes (or until the 10% bile salt reagent dries).
   - *Optional:* instead of leaving the plate out at room temperature, laboratorians may choose to put the plate top-side (*i.e.*, agar-side) up on a level surface in an ambient air incubator (*i.e.*, **not** a CO_2-incubator) at 35˚C until the reagent dries (approximately 10–15 minutes).

c) When the reagent dropped on the suspect colony is dry, read, record, and interpret the results.

Pneumococcal colonies are bile-soluble and will disappear or appear as flattened colonies; in contrast, bile-resistant streptococcal colonies will be unaffected.

Interpretation of the combined optochin and bile solubility tests for pneumococcal identification

The following summary of results of the optochin and bile-solubility tests is commonly used to accurately and conveniently identify *S. pneumoniae* (*i.e.*, pneumococcus).

- A strain exhibiting a zone of inhibition by optochin ≥14 mm (with a 6-mm, 5-µg disk) is a pneumococcus.
- A strain exhibiting a smaller but definite zone of inhibition by optochin (9–13 mm with a 6-mm, 5-mg disk) **and** that is also bile soluble is a pneumococcus.

The following summary of results of the optochin and bile-solubility tests should be interpreted as negative for *S. pneumoniae* (and positive for viridans streptococci).

- A strain with a small zone of inhibition by optochin (≤8 mm with a 6-mm, 5-µg disk) that is not bile soluble is not a pneumococcus. (The colonies can be identified as viridans streptococci.)
- Strains with no zones of inhibition by optochin are not pneumococci. (The colonies can be identified as viridans streptococci.)

Commercial test kits for identification (slide agglutination test)

Commercially available slide agglutination tests (*e.g.*, Slidex Pneumo-kit® and the Pneumoslide™) can also help identify colony growth from blood agar plates as *S. pneumoniae*. Follow the manufacturer's instructions precisely when using these and any other commercial tests.

If a colony appears to be *S. pneumoniae* on the basis of morphology and susceptibility to optochin, but it has a negative bile solubility test, slide agglutination tests can assist with identification of the isolate. A positive slide agglutination test should be interpreted as a possible *S. pneumoniae* isolate, whereas a negative slide agglutination reaction in conjunction with the positive optochin and negative bile solubility would indicate the isolate is not *S. pneumoniae*.

Identification of the S. pneumoniae *serotype*

Serotyping of pneumococci is not usually necessary for a clinical response. However, in some situations or settings (*e.g.*, studies focusing on evaluation

of vaccine efficacy), it will be appropriate to type these isolates. Methods for serotyping and Quellung typing are included in Appendix 6.

Antimicrobial susceptibility testing of *S. pneumoniae*

The results of antimicrobial susceptibility tests will be used to help make recommendations for clinical treatment. There are a variety of methods by which one can determine the antimicrobial susceptibility of a bacterial pathogen, commonly including disk diffusion, testing by agar dilution or broth microdilution, and testing by antimicrobial gradient agar diffusion (*e.g.*, with the Etest® strip). The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS;[13] if performed precisely according to the following protocol, this method will yield data that can reliably predict the *in vivo* effectiveness of the drug in question. This section describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results for *S. pneumoniae.*

The disk diffusion method gives valid data for only certain antibiotics, so this laboratory manual recommends use of the Etest® to gather data about the minimal inhibitory concentration (MIC) of antimicrobial agents.[14] MIC testing can also be done by dilution; however because agar dilution and broth microdilution are expensive and technically complex, this manual recommends that countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. (Alternatively, if resources are available, laboratories may purchase commercially-available, frozen MIC panels and follow the manufacturer's instructions to carry out the MIC test.)

This laboratory manual describes antimicrobial susceptibility testing of *S. pneumoniae* by the disk diffusion method and by the Etest® antimicrobial gradient strip method. (Figure 18 is a sample worksheet for recording results of the antimicrobial susceptibility tests.) Although disk diffusion will provide information for most antimicrobial agents regarding interpretation of a strain as susceptible, intermediate, or resistant, the Etest® provides general information about the MIC of antibiotic. **The accuracy and reproducibility of this test are dependent on following a standard set of procedures and conditions in laboratories on an on-going basis.**

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[13] Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

[14] The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

FIGURE 18: Sample form for recording antimicrobial susceptibility test results for ***Streptococcus pneumoniae***

Date of Testing: ____/____/_____
*Test performed by:*_____

Interpretation of susceptibility: *S* = susceptible *I* = intermediate *R* = resistant

Specimen number	Meningitis isolate?[a]	Organism	Chloramphenicol	Trimethoprim-sulfamethoxazole	Oxacillin[b] *(disk) or* (Penicillin) *(MIC)*	*(other drug)* _____
			mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*
			mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*
			mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*	mm µg/ml *S I R*
ATCC 49619	N/A	NCCLS QC strain *QC in range? →*	mm µg/ml *Yes No*	mm µg/ml *Yes No*	mm µg/ml *Yes No*	mm µg/ml *Yes No*

[a] if an *S. pneumoniae* isolate is from a meningitis patient, the breakpoints for interpretation of the MIC may differ from those for isolates from other sites.
[b] if an oxacillin disk yields a zone diameter <20 mm for *S. pneumoniae*, MIC testing to a specific penicillin must be done in order to interpret the susceptibility.

Reveiwed by: _____ ***Date of Report:*** ____/____/_____

Note: After 20-24 hours of incubation, check the results for the quality control (QC) strains against the standard acceptable ranges; if they are within control limits, continue reading results for the test isolate. Record disk diffusion results in mm and MIC results in µg/ml. *(Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 5.)*

Quality control of antimicrobial susceptibility testing of *S. pneumoniae*

Quality control tests must be performed as part of the normal laboratory routine. To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test or new set of testing conditions. *S. pneumoniae* ATCC 49619 is the NCCLS control strain to use when performing antimicrobial susceptibility testing on *S. pneumoniae* isolates. **Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits, which are included in Table 5.** If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

- **Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other environmental factors.** The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not.
- **If the depth of the agar in the plate is not uniformly 3–4 mm,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
- **If the pH of the test medium is not between 7.2 and 7.4,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected. (***Note:*** Do not attempt to adjust the pH of the Mueller-Hinton agar test medium if it is outside the range; see Appendix 2.)
- **If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected.** For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, even if the isolates are susceptible, when colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks.

Quality control ("QC") tests should be performed once per week if antimicrobial susceptibility tests are performed daily after 30 days of in-control results, or with every group of tests when testing is done less frequently. They should also be done with each new batch of antimicrobial susceptibility test medium and every time a new lot of disks is used.

Antimicrobial susceptibility testing by disk diffusion

Antimicrobial susceptibility can be determined using the disk diffusion method; however, disk diffusion antimicrobial susceptibility testing is generally not performed on meningitis isolates. This laboratory manual describes the optimal media, inoculum, antimicrobial agents to be tested, incubation conditions, and interpretation of results.

- Mueller-Hinton agar medium supplemented with 5% sheep blood is recommended for determining the antimicrobial susceptibility of *S. pneumoniae* specimens by disk diffusion. The agar plates should have a uniform depth of 3–4 mm.
- The l-µg oxacillin disk is recommended for predicting the susceptibility of *S. pneumoniae* to penicillin because penicillin disks do not provide reproducible results. Interpretations of the oxacillin disk diffusion test are generalizable across the ß-lactam drugs for *S. pneumoniae.*
  - **It is only possible to conclude if a strain is susceptible to penicillin based on the oxacillin screen,** and **not** if it is resistant to penicillin. **If the zone of inhibition around the oxacillin disk is less than 20 mm, additional MIC testing** (*e.g.*, by Etest®) **must be performed to assess whether the isolate is resistant or susceptible** to penicillin.
- A 30-µg chloramphenicol disk is used for detecting resistance to chloramphenicol.
- A 25-µg trimethoprim-sulfamethoxazole (cotrimoxazole) disk (*i.e.*, a disk comprised of 1.25 mg trimethoprim plus 23.75 mg sulfamethoxazole) is used for detecting trimethoprim-sulfamethoxazole resistance in *S. pneumoniae.* **The Mueller-Hinton agar used for this test should be thymidine free to obtain accurate results with trimethoprim-sulfamethoxazole.**

Methods for antimicrobial susceptibility testing by disk diffusion

Prepare the inoculum for antimicrobial susceptibility testing of *S. pneumoniae* from fresh pure cultures of *S. pneumoniae* (grown overnight on blood or chocolate agar). Prepare cell suspensions of the bacteria to be tested in sterile physiological saline or Mueller-Hinton broth. A cell suspension equal to a density of a 0.5 McFarland turbidity standard is used for the inoculum. (Preparation of a McFarland turbidity standard and plate count methods are described in Appendix 2.)

a) Suspend viable colonies from an overnight sheep blood or chocolate agar plate in a tube of broth to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells with the broth. **This suspension should be used within 15 minutes.**

b) Compare the density of the suspension to the 0.5 McFarland turbidity standard by holding the suspension and McFarland turbidity standard in front of a light against a white background with contrasting black lines (see Figures 51 and 52, Appendix 2). If the density is too heavy, the suspension should be diluted with additional suspending medium (*i.e.*, saline or broth). If the density is too light, additional bacteria should be added to the suspension.

c) When the proper density is achieved, dip a cotton or dacron swab into the bacterial suspension. Lift it out of the broth and remove excess fluid by pressing and rotating the swab against the wall of the tube.

d) Use the swab to inoculate the entire surface of the supplemented Mueller-Hinton agar plate three times, rotating the plate 60 degrees between each inoculation (see Figure 34). Use the same swab with each rotated streak, but **do not re-dip the swab in the inoculum** (*i.e.*, the bacterial cell suspension).

e) Allow the inoculum to dry before placing the disks on the plates. Drying usually takes only a few minutes, and should take no longer than 15 minutes. (If drying takes longer than 15 minutes, use a smaller volume of inoculum in the future.)

f) After the plate is dry, place the antimicrobial disks on the plates (as shown in Figure 6). Use sterile forceps to place the disks on the Mueller Hinton agar and tap them gently to ensure they adhere to the agar. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.**

g) Incubate the plates in an inverted position in a 5% CO_2 atmosphere for 20–24 hours at 35°C. A candle-extinction jar may be used if a CO_2-incubator is not available.

- If this is a new batch of Mueller-Hinton agar, the antimicrobial disks are new, or it is an otherwise appropriate time to perform quality control, follow steps a through g above and run parallel tests on the reference strain(s). Appropriate disk diffusion zone sizes for the reference QC strains are included in Table 5.

h) After overnight incubation, measure the diameter of each zone of inhibition with a ruler or calipers. The zones of inhibition on the media containing blood are measured from the top surface of the plate with the top removed. Use either calipers or a ruler with a handle attached for these measurements, holding the ruler over the center of the surface of the disk when measuring the inhibition zone (see Figure 6).

- Care should be taken not to touch the disk or surface of the agar. Sterilize the ruler occasionally to prevent transmission of the bacteria. In all measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony. Record the results in millimeters (mm). Figure 5 provides a sample form for recording results.

TABLE 5: Antimicrobial susceptibility test breakpoints and quality control ranges for ***Streptococcus pneumoniae***

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml) [a]			NCCLS QC strain ***S. pneumoniae*** ATCC 49619
		Susceptible	Intermediate	Resistant	
Chloramphenicol	30 µg	≥ 21 mm *(≤ 4 µg/ml)*	~ ~	≤ 20 mm *(≥ 8 µg/ml)*	23 – 27 mm *(2 – 8 µg/ml)*
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 µg	≥ 19 mm *(≤ 0.5 – 9.5 µg/ml)*	16 – 18 mm *(1/19 – 2/38 µg/ml)*	≤ 15 mm *(≥4/76 µg/ml)*	20 – 28 mm *(0.12/2.4 – 1/19 µg/ml)*
Oxacillin [b]	1 µg Disk diffusion ONLY	≥ 20 mm [b]	** [b]	** [b]	≤ 12mm [c]
Penicillin [b]	MIC ONLY	*(≤ 0.06 µg/ml)*	*(0.12 – 1 µg/ml)*	*(≥ 2 µg/ml)*	*(0.25 – 1 µg/ml)*
Ceftriaxone [d, e]	MIC ONLY *Non-meningitis isolate MIC*	*(≤ 1 µg/ml)*	*(2 µg/ml)*	*(≥ 4 µg/ml)*	*(0.03 – 0.12 µg/ml)*
	Meningitis isolate MIC	*(≤ 0.5 µg/ml)*	*(1 µg/ml)*	*(≥ 2 µg/ml)*	
Cefotaxime [d, e]	MIC ONLY *Non-meningitis isolate MIC*	*(≤ 1 µg/ml)*	*(2 µg/ml)*	*(≥ 4 µg/ml)*	*(0.03 – 0.12 µg/ml)*
	Meningitis isolate MIC	*(≤ 0.5 µg/ml)*	*(1 µg/ml)*	*(≥ 2 µg/ml)*	

[a] *Source:* NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA.

[b] Oxacillin should only be tested by disk diffusion. If the zone is <20 mm, an isolate cannot be reported as susceptible, intermediate or resistant, and MIC testing must be conducted for an appropriate penicillin (or other ß-lactam) drug.

[c] "Deterioration in oxacillin disk content is best assessed with QC organism *Staphylococcus aureus* ATCC 25923, with an acceptable zone diameter of 18 to 24 mm" [NCCLS 2002].

[d] Penicillin, ceftriaxone, and cefotaxime should only be tested by a method that will provide an MIC; it is presented in this table as a follow-up for an equivocal oxacillin disk diffusion test (*i.e.*, oxacillin zone of inhibition <20 mm). Perform MIC testing on the specific penicillin (or other ß-lactam) drug that would be used to treat.

[e] Ceftriaxone and cefotaxime have separate interpretive MIC breakpoints for meningitis and non-meningitis isolates.

i) Interpret the antimicrobial susceptibility of the test strain (and check that results for the QC strain *S. pneumoniae* ATCC 49619 are within the acceptable control range) by comparing the results to the NCCLS standard zone sizes (Table 5).

The Etest® for minimal inhibitory concentration testing of *S. pneumoniae*

For *S. pneumoniae*, disk diffusion testing indicates whether an organism is susceptible or resistant to an antimicrobial for most agents. However, disk diffusion testing for pneumococcal isolates and oxacillin (a penicillin agent) is not sufficient to distinguish between complete and intermediate resistance. For surveillance purposes, a laboratory may want to quantify the results of the oxacillin

disk diffusion test by performing minimal inhibitory concentration (MIC) testing of penicillin or any other beta-lactam antibiotic that would be used for treatment. As mentioned earlier in this manual, MIC testing by dilution can be expensive and challenging, and because of the technical complexity required for these tests, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. The World Health Organization (WHO) recommends that only one laboratory in a resource-limited region perform antimicrobial susceptibility testing; however, in countries where MIC testing is done at more than one laboratory, standardization and quality control should be conducted at each laboratory in accordance with the standardized guidelines presented in this manual.

Laboratorians determining the minimal inhibitory concentration (MIC) for resistant isolates must be highly skilled in performing these tests and committed to obtaining accurate and reproducible results. In addition, a national (or regional) reference laboratory must have the ability and resources to store isolates either by lyophilization or by freezing at -70˚C. Methods for preservation and storage of isolates are presented in Appendix 11, and detailed methods for transport of isolates according to international regulations are presented in Appendix 12.

With increasing antimicrobial resistance testing being performed outside of international reference laboratories, the Etest® serves as a test method that is both convenient and reliable.[15] The Etest® requires less technical expertise than MIC testing by dilution methods, but it gives comparable results. **Etest® strips must be consistently stored in a freezer at -20˚C.**

The Etest® is an antimicrobial susceptibility testing method that is as technically simple to perform as disk diffusion and produces semi-quantitative results that are measured in micrograms per milliliter (μg/ml). It is drug-specific, consists of a thin plastic antibiotic gradient strip that is applied to an inoculated agar plate, and is convenient in that it applies the principles of agar diffusion to perform semi-quantitative testing.[16]

The continuous concentration gradient of stabilized, dried antibiotic is equivalent to 15 $\log_2$ dilutions by a conventional reference MIC procedure as suggested by the NCCLS. The Etest® has been compared and evaluated beside both the agar and broth dilution susceptibility testing methods recommended by the NCCLS. Authoritative reports indicate that an (approximately) 85% – 100% correlation exists between the accepted conventional MIC determinations and the MIC determined by the Etest® procedure for a variety of organism-drug combinations

[15] The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

[16] Antimicrobial susceptibility testing with an antimicrobial gradient strip such as the Etest® can be considered to be a semi-quantitative method (because although the suspension used to inoculate a plate for Etest® is standardized, the inoculum itself is not standardized). However, results are generally comparable to quantitative results of standard broth microdilution or agar dilution MIC tests.

(see, *e.g.,* Jorgensen *et al.* [1994] and Barry *et al.* [1996] in Appendix 15). Some studies have cited Etest® MICs as approximately one dilution higher than MICs determined by standard dilution methods.

Although this manual serves as a general guide to use of the Etest® antimicrobial gradient strip, **always follow the manufacturer's directions for use of the Etest®,** as certain antibiotic-bacteria ("drug-bug") combinations have special testing requirements. For example, macrolides (*e.g.*, azithromycin, erythromycin) should be tested in a normal atmosphere, not with CO_2.

Methods for performing antimicrobial susceptibility testing of S. pneumoniae *with the Etest®*

The manufacturer of the Etest® indicates that when testing *S. pneumoniae*, the Mueller-Hinton agar test medium can be supplemented with either sheep or horse blood; however, it may be easier to interpret results on medium prepared with sheep blood (except when testing susceptibility to trimethoprim-sulfamethoxazole, in which case sheep blood should not be used as a supplement) [CDC, unpublished data]. This laboratory manual therefore suggests that Mueller Hinton agar with 5% sheep blood should be used when performing antimicrobial susceptibility testing of *S. pneumoniae* with the Etest® (except when testing for susceptibility to **trimethoprim-sulfamethoxazole, in which case horse blood should be used** in place of sheep blood). Either 150-mm or 100-mm plates can be used, depending on the number of Etests® used per sample (Figure 7). Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and note that although the manufacturer states that up to six Etest® strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five Etest® strips be used on a 150-mm plate.

a) Suspend viable colonies from an overnight blood agar plate into a broth tube to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells. **This suspension should be used within 15 minutes.**

b) Dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid. Inoculate the entire surface of the agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure confluent growth of the bacteria (Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.

c) Allow the plate to dry for up to 15 minutes. **Be sure the plate is entirely dry before proceeding.** While the plate is drying, remove the Etest® strips from the -20˚C freezer, and allow the strips that will be used in the batch of testing to warm to room temperature. Return the strips that will not be used in this batch of testing to the -20˚C freezer.

d) Place the Etest® strips onto the dried, inoculated agar plate with an Etest® applicator or sterile forceps (Figure 7.) Make sure that the printed MIC values are facing upward (*i.e.*, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar.) **Once applied, do not move the antimicrobial gradient strips.**

e) Incubate the plates in an inverted position in a CO_2-enriched atmosphere (2%–5% CO_2) for 20–24 hours at 35°C. A candle-extinction jar may be used if a CO_2 incubator is not available.

- Always follow the manufacturer's instructions included with each package of strips, because incubation conditions may vary by organism-antimicrobial (or "drug-bug") combination.

f) After incubation, there will be an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **It is important to review quality control results before reading and interpreting the Etest® MIC.**

MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip. Use oblique light to carefully examine the end-point. A magnifying glass may be used if needed. Read at the point of complete inhibition of all growth including hazes and isolated colonies. Figure 8 presents a reading guide for the Etest®,[17] and shows drug-related effects, technical and handling effects, organism-related effects and resistance-mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but the marks also represent increments between those standard values. The standard values (Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that if the MIC appears to be an inter-dilutional value, both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of a *S. pneumoniae* isolate to penicillin, an MIC recorded from the graduations on the Etest® strip might be 0.094 μg/ml; however, the reported MIC would be 0.125μg/ml, and the organism would be interpreted as being intermediate to penicillin.

Breakpoints follow the NCCLS guidelines, **unless exceptions made by the manufacturer are provided in the package insert.** NCCLS breakpoints for *S. pneumoniae*–antimicrobial combinations are included in Table 5.

[17] AB Biodisk also maintains a website with an Etest® reading guide: http://www.abbiodisk.com.

Surveillance for emerging pneumococcal resistance

Laboratories may wish to help detect the emergence of new strains of pathogens by testing isolates against a panel of drugs in which reduced susceptibility is not expected to be found. This could be done, for example, on a sampling of preserved isolates in storage on an annual basis. Methods for preservation and long-term storage of isolates can be found in Appendix 11.

Antimicrobials of interest could include (but are not necessarily limited to): tetracycline, erythromycin, clindamycin, rifampin, ceftriaxone, amoxicillin, ciprofloxacin and vancomycin. Appropriate zone sizes can be found in NCCLS documents, which are updated regularly. Laboratorians should notify a reference laboratory of any isolates observed to have rare characteristics of non-susceptibility; for example, as of early 2002, no pneumococcus has exhibited decreased susceptibility to vancomycin [NCCLS 2002]. A list of international reference laboratories is included in Appendix 14.

Data for decision-making

Once the laboratory has antimicrobial susceptibility patterns of *S. pneumoniae* isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and the specific antimicrobial susceptibility profile. National recommendations for empiric antibiotic utilization should be developed after considering antimicrobial susceptibility data, cost, availability, convenience, and other factors. Information on pneumococcal antimicrobial resistance, together with data on the major pneumococcal serotypes responsible for disease, may become increasingly valuable to public health officials in the future, as new formulations of multivalent pneumococcal conjugate vaccines become available for global use.[18]

[18] The Vaccine Alliance maintains information on these sorts of activities on its website: www.vaccinealliance.org.

Sexually Transmitted Bacterial Pathogen for which there are Increasing Antimicrobial Resistance Concerns

Neisseria gonorrhoeae

CHAPTER VI

Neisseria gonorrhoeae

CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Neisseria gonorrhoeae, also commonly referred to as "gonococcus" or "GC", causes an estimated 62 million cases of gonorrhea worldwide each year [Gerbase *et al.*, 1998]. Spread by sexual intercourse, *N. gonorrhoeae* may infect the mucosal surfaces of urogenital sites (cervix, urethra, rectum) and the oro- and nasopharynx (throat), causing symptomatic or asymptomatic infections. GC is always pathogenic and, if untreated, gonorrhea is a major cause of pelvic inflammatory disease (PID), tubal infertility, ectopic pregnancy, chronic pelvic pain and/or disseminated gonococcal infection (DGI). The probability of co-infection with other sexually transmitted infections (STIs) may be high in some patient populations. Neonates may acquire gonococcal infection of the conjunctiva during birth. The diagnosis of gonorrhea in older infants and young children is often associated with allegations of sexual abuse; transmission through neither nonsexual human nor fomite contact has been documented. Epidemiological studies provide strong evidence that gonococcal infections facilitate HIV transmission [Fleming and Wasserheit 1999]. Extended-spectrum cephalosporins, fluoroquinolones and spectinomycin are recognized as the most effective antibiotics for the treatment of gonorrhea in most areas of the world.

Antimicrobial resistance in *N. gonorrhoeae* is the most significant challenge to controlling gonorrhea. Gonococcal strains may be resistant to penicillins, tetracyclines, spectinomycin, and, recently, resistance to the fluoroquinolones (ciprofloxacin and ofloxacin) and the macrolide azithromycin has emerged [Handsfield 1994; Knapp *et al.* 1997; Young *et al.* 1997; CDC 1999]. Resistance to the penicillins and tetracyclines is conferred by chromosomal and/or plasmid-mediated mechanisms. Resistance to spectinomycin, fluoroquinolones and azithromycin is chromosomally mediated, and certain types of chromosomal mutations may contribute to resistance to several classes of antibiotics simultaneously.

Agents used for the treatment of bacterial infections, including co-infecting STIs, may select for resistance in *N. gonorrhoeae*. For example, whereas a 1-gram dose of azithromycin is sufficient for treatment of infections with *C. trachomatis* and *H. ducreyi*, this dose is sub-optimal for the treatment of *N. gonorrhoeae* and may result in the incidental selection and spread of resistant gonococcal strains. At the time of writing of this manual (2002), the broad-spectrum cephalosporins (ceftriaxone, cefixime, etc.) are the only class of antimicrobial agents to which gonococci have not developed confirmed resistance, although a few isolated strains have exhibited decreased susceptibility to cefixime [CDC 2000; Wang 2002].

It is of great importance to perform laboratory surveillance of antimicrobial resistance in *N. gonorrhoeae* in order to assess the effectiveness of locally recommended therapies. Only measurement of the *in vitro* susceptibilities of the infecting organism will provide objective information to help determine if a post-treatment isolate is truly resistant to the antimicrobial agent being used to treat the infection, as opposed to infection which fails to respond to treatment due to inadequate absorption of the agent, non-compliance with therapy, or re-exposure. At the population level, surveillance is key for the monitoring of local, regional and international trends in antimicrobial resistance, which can help inform and shape public health policy. Comparison between antimicrobial susceptibilities of gonococci isolated in different geographical areas provides information about the distribution and temporal spread of resistant isolates. Thus, changes in recommended antimicrobial therapies can be anticipated, and surveillance can be enhanced to guide timely changes in these therapies at the local level.

Presumptive identification of *N. gonorrhoeae*

After the specimen has been collected from the patient, it should be labeled with a unique identifier assigned in tandem with the demographic and clinical information so it can be linked for epidemiological studies. Methods for streaking for isolation from specimen swabs, primary culture methodology, and isolate storage and transport are included in Appendices 8, 11 and 12.

Because *N. gonorrhoeae* is highly susceptible to adverse environmental conditions (as described in Table 28 of Appendix 8), strains must always be incubated at 35˚–36.5˚C in a humid, CO_2-enriched atmosphere. Subculture colonies that appear to be gonococcal (gram-negative diplococci growing in pinkish-brown colonies 0.5 – 1 mm in diameter, see Appendix 8) from the primary selective medium to a non-selective medium, such as GC-chocolate agar with 1% defined supplement, to obtain a pure culture of the isolate. (Specimens from normally sterile sites, such as the conjunctiva, are cultured on nonselective medium for primary isolation; subculture for purity if examination of the plate shows evidence of contaminants.) If the subcultured isolate is not pure, continue to perform serial subcultures of individual colonies of gram-negative diplococci until a pure culture is obtained.

A presumptive diagnosis of *N. gonorrhoeae* originally isolated on selective medium can be made based upon colonial morphology, the observation of typical (gram-negative) diplococci in pairs, tetrads or clusters upon Gram stain or simple single stain with Loeffler's methylene blue, and a positive oxidase reaction. A presumptive diagnosis of *N. gonorrhoeae* originally isolated on nonselective medium can be made based upon these characteristics **plus** an appropriate reaction in at least one supplemental biochemical or enzymatic test (*e.g.*, superoxol 4+ reaction, see 'Supplemental Tests'). A flowchart of tests required for presumptive identification of isolates from sites with normal flora (*i.e.*, isolated on selective media such as MTM, ML, or GC-Lect) and isolates from normally sterile sites (*i.e.*, isolated on nonselective medium, such as GC-chocolate agar) is presented in Figure 19.

Oxidase test

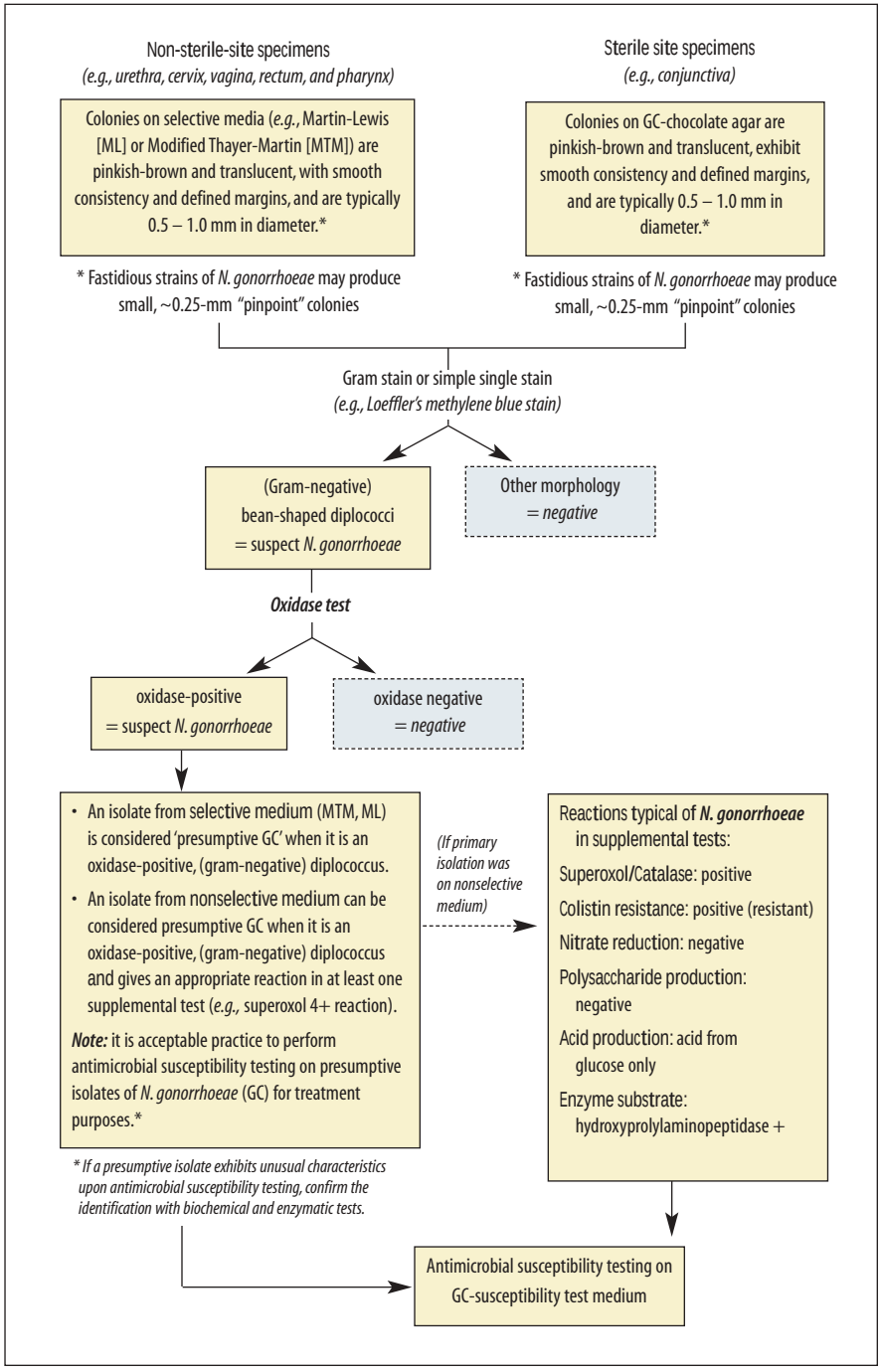
The oxidase test uses Kovac's reagent (a 1% (wt/vol) solution of *N, N, N', N'* –tetramethyl-ρ-phenylenediamine dihydrochloride)[18] to detect the presence of cytochrome c in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. *Neisseria* species give a positive oxidase reaction, and gram-negative oxidase-positive diplococci isolated on gonococcal selective media may be identified presumptively as *N. gonorrhoeae*. Preparation of oxidase reagent and appropriate quality control methods are included in Appendix 2.

Perform an oxidase test on growth of representative colonies that stained as (gram-negative) diplococci. Because the oxidase reagent is toxic for bacteria, it is recommended to perform the oxidase test on a sterile swab and not directly on the culture plate, particularly if there are only a few suspect colonies. Alternatively, one can use filter paper in place of a swab for this test. **Do not perform the oxidase test with a Nichrome loop,** as it may produce a false-positive reaction. If a sterile swab was used to make a smear for the Gram stain (as described in Appendix 4), the swab can then be used to conduct the oxidase test. The oxidase test should only be performed on freshly grown (18–24 hour) organisms.

- ***Swab method for Kovac's oxidase test***
  a) Select suspect colonies from the culture plate (selective or nonselective medium) with the swab.
  b) Use a Pasteur pipette to add one drop of oxidase reagent to the swab.
  c) If the isolate is *N. gonorrhoeae*, a positive (purple) reaction should occur within 10 seconds.[18] (See Figure 20).

[18] Some laboratories may use a different reagent, Gordon and MacLeod's reagent, (1% [wt/vol] dimethyl-ρ-phenylenediamene dihydrocholoride; "dimethyl reagent") to perform the oxidase test. The dimethyl reagent is more stable than the tetramethyl reagent (Kovac's reagent), but the reaction with the dimethyl reagent is slower than that with the tetramethyl reagent. **If the laboratory is using the dimethyl- reagent,** a positive reaction will be indicated by a color change to blue on the filter paper (not purple, as with the tetramethyl reagent), and **with the dimethyl reagent it will take 10 – 30 minutes for a positive reaction to develop.**

FIGURE 19: Flowchart for isolation and presumptive identification of ***Neisseria gonorrhoeae***



- ***Moistened filter paper method for Kovac's oxidase test***
  a) Place a piece of filter paper in a petri dish.
  b) Just prior to performing the test, add one to two drops of oxidase reagent to the filter paper and allow it to absorb; the filter paper should be moist, but not wet, after the reagent has been absorbed.
  c) Using a platinum loop, a plastic loop, a sterile swab or a wooden applicator stick, pick a portion of the colony to be tested and rub it onto the moistened filter paper. (**Do not use a Nichrome loop.**) If the isolate is *N. gonorrhoeae*, a positive (purple) reaction should occur within 10 seconds.[18] (See Figure 10.)

Confirmatory identification of *N. gonorrhoeae*

If a laboratory is reporting results back to the clinical setting for treatment purposes, a presumptive diagnosis based on Gram stain and oxidase reaction is sufficient for colonies isolated on GC-selective media, and the laboratorian can continue with antimicrobial susceptibility testing of a pure culture of the isolate (presented later in this chapter). If, however, the diagnosis must be confirmed or a presumptive isolate exhibits unusual characteristics upon antimicrobial susceptibility testing (*e.g*, for ceftriaxone, a minimal inhibitory concentration (MIC) >0.25µg/ml, or equivalent inhibition zone diameter <35mm), the laboratorian should perform biochemical and enzymatic tests of pure culture to confirm the identification of the isolate. It is worth noting, for example, that

FIGURE 20: Kovac's oxidase test: a positive reaction on a swab

The right-hand picture shows a positive reaction on a swab that was used to harvest suspect growth and was then moistened with Kovac's oxidase reagent. The left-hand picture shows a positive oxidase direct-plate test result with Kovac's oxidase. Note that if growth is sparse, it is suggested that a laboratory **not** use the direct-plate testing method because it is toxic to gonococcal growth.

because men who have sex with men (referred to in literature as "MSM") have higher rates of non-gonococcal neisserial infections in the urethra than do other populations, the epidemiology could lead a clinician to request a confirmed diagnosis. Another example of a situation where the diagnosis requires definitive confirmation would be a case of suspected sexual abuse; the discussion of the related social, medical and legal issues with which a laboratory could be involved goes beyond the scope of this laboratory manual.[19]

Figure 21 shows one pathway by which diagnosis might be confirmed with biochemical and enzymatic tests. This laboratory manual will present methods to perform tests for a reaction to superoxol reagent (or catalase reagent), colistin resistance, the production of polysaccharide from sucrose, detection of acid production with a commercial test, detection of enzyme production by a chromogenic substrate in a commercial test, and nitrate reduction. Table 6 provides a listing of reactions to a variety of tests performed on non-gonococcal species which may be mistakenly identified as *N. gonorrhoeae* based on reactions only with the acid production or enzyme substrate tests. The table includes a blank row so it may be copied and used as a sample worksheet in which to record results of confirmatory tests.

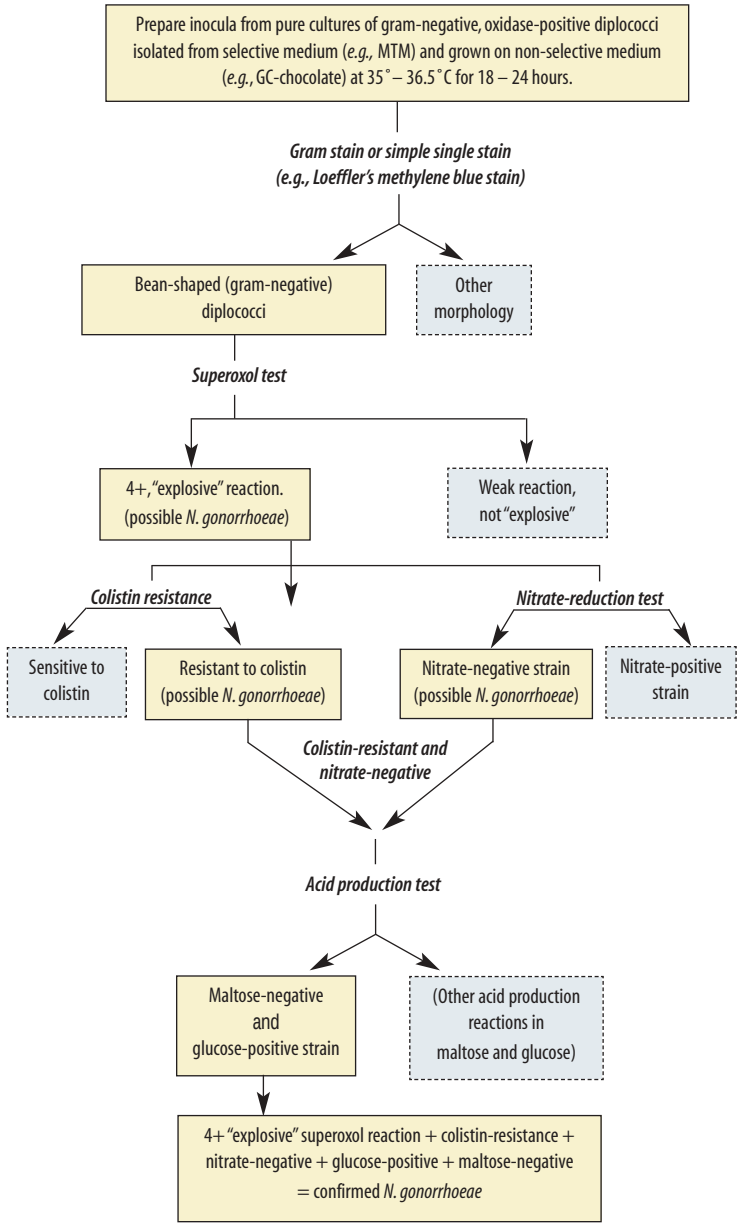
Laboratorians wishing to learn more about the concepts behind the biochemical and enzyme substrate test reactions presented here, or seeking information about other tests and methodologies in more detail, can refer to the American Society of Microbiology's *Manual of Clinical Microbiology,* or, for example, to the CDC website for clinical diagnosis of gonorrhea (*http://www.cdc.gov/ncidod/dastlr/gcdir/gono.html*).

Biochemical and enzyme substrate supplemental tests

Species of three genera—*Neisseria*, *Kingella*, and *Moraxella* (*Branhamella*)—must be considered when examining clinical specimens or cultures for *N. gonorrhoeae*. *Neisseria* species (except *N. elongata* and *N. weaveri*) and *M. catarrhalis* are gram-negative diplococci and, in stained smears, resemble *N. gonorrhoeae*, exhibiting kidney bean- or coffee bean-shaped diplococci with adjacent sides flattened. It should be noted that is not unusual to isolate *N. meningitidis* from urethral specimens from men who have sex with men or to isolate *N. lactamica* from the throats of young children. *Kingella denitrificans* and *Moraxella* species are coccobacilli, but cells of some strains may occur as pairs and look like diplococci in smears. Thus, all of these species must be considered when identifying gram-negative diplococci in clinical specimens. Characteristics that differentiate among these genera and species are presented in Appendix 8 and Table 6. A sample listing

[19] The Centers for Disease Control and Prevention (CDC) maintains a website that includes information regarding social, medical and legal issues surrounding the diagnosis of gonorrhea and with which a public health laboratory might become involved. See: *http://www.cdc.gov/ncidod/dastlr/gcdir/NeIdent/Ngon.html#Medicolegal* .

FIGURE 21: Flowchart exhibiting one algorithm for confirmatory identification of ***Neisseria gonorrhoeae***



Note: if resources are available, several confirmatory tests may be run concurrently, rather than waiting for results from each test before continuing.

TABLE 6: Results of biochemical and enzymatic tests for ***Neisseria gonorrhoeae*** and related species with similar colonial morphology

Species	Cell morphology	Superoxol *{Catalase}*	Colistin	Production of acid from: GLU	MAL	LAC	SUC	Reduction of NO_3 (Nitrate)	Polysaccharide from sucrose
Test isolate:									
N. gonorrhoeae [a]	GND	4+ *{+}*	R	+	–	–	–	–	–
N. meningitidis	GND	1+ to 4+ *{+}*	R	+	+	–	–	–	–
N. lactamica	GND	1+ to 3+ *{+}*	R	+	+	+	–	–	–
N. cinerea [b]	GND	2+ *{+}*	(R)	–	–	–	–	–	–
N. polysaccharea	GND	1+ to 3+ *{+}*	(R)	+	+	–	–	–	–
N. subflava [b]	GND	2+ *{+}*	(R)	+	+	–	V	–	V
N. sicca	GND	2+ *{+}*	S	+	+	–	+	–	+
N. mucosa	GND	2+ *{+}*	S	+	+	–	+	+	+
N. flavescens	GND	2+ *{+}*	S	–	–	–	–	–	+
N. elongata	GNR	– *{–}*	S	–	–	–	–	–	–
M. catarrhalis	GND	1+ to 4+ *{+}*	(R)	–	–	–	–	+	–
K. denitrificans [c]	GNC	– *{–}*	R	+	–	–	–	+	–

Symbols and Abbreviations: **+**, strains typically positive but genetic mutants may be negative; **–** , strains typically negative; **V**, biovar dependent (strains belonging to biovars flava and subflava do not produce acid from sucrose or produce polysaccharide from sucrose); **GLU**, glucose; **MAL**, maltose; **LAC**, lactose; **SUC**, sucrose; **GND**, gram-negative diplococci; **GNR**, gram-negative rods; **GNC**, gram-negative coccobacilli; **R**, resistant; (**R**), some strains resistant and may grow on gonococcal selective media; **S**, susceptible (insufficient data to suggest that isolates may grow on gonococcal selective media containing colistin).

[a] Includes *N. gonorrhoeae* subspecies kochii which exhibit characteristics of both *N. gonorrhoeae* and *N. meningitidis* (but will be identified as *N. gonorrhoeae* by tests routinely used for the identification of *Neisseria* species).

[b] Includes biovars subflava, flava, and perflava. Strains belonging to the biovar flava produce acid from glucose, maltose and fructose; strains belonging to the biovar subflava produce acid only from glucose and maltose.

[c] Coccobacillus; some strains occur in pairs which resemble gram-negative diplococci

of quality control strains for the supplemental tests described in this manual for the identification of *N. gonorrhoeae* is included in Table 7.

In a reference laboratory setting, the tests described below are best performed concurrently since they all require an inoculum prepared from fresh (18–24 hour) growth. However, when resources are limited, laboratorians may choose to screen isolates with a subset of these tests to detect isolates resembling *N. gonorrhoeae* prior to further testing. Sequential testing practices can conserve resources by limiting the use of more costly commercial tests (*e.g.*, acid production or enzyme substrate) to only those isolates resistant to colistin and exhibiting a strong superoxol reaction. When choosing the screening approach, it is important to remember that tests performed on successive days will require a fresh (18–24 hour) subculture of the isolate.

TABLE 7: Examples of quality control (QC) strains for supplemental tests used to identify ***Neisseria gonorrhoeae***

Test	Positive control	Negative control
Superoxol (or Catalase) test	*N. gonorrhoeae* ATCC 49226 [4+] *N. cinerea* ATCC 14685 [weak, 2+] (positive reaction in superoxol)	*K. denitrificans* ATCC 33394 (no reaction in superoxol)
Colistin resistance test	*N. gonorrhoeae* ATCC 49226 *K. denitrificans* ATCC 33394 (resistant to colistin)	*N. cinerea* ATCC 14685 *N. mucosa* ATCC 19696 (susceptible to colistin)
Polysaccharide production test	*N. polysaccharea* ATCC 43768 *N. mucosa* ATCC 19696 (produce polysaccharide)	*N. gonorrhoeae* ATCC 49226 *N. cinerea* ATCC 14685 (do not produce polysaccharide)
Nitrate reduction test	*K. denitrificans* ATCC 33394 *N. mucosa* ATCC 19696 (able to reduce nitrate)	*N. gonorrhoeae* ATCC 49226 *N. cinerea* ATCC 14685 (unable to reduce nitrate)
Acid production test	Use the QC strains recommended by the test manufacturer* plus *N. cinerea*. * If the manufacturer has not designated specific strains for QC: • *N. gonorrhoeae* (ATCC 49226) produces acid from glucose • *N. meningitidis* (ATCC 13077) produces acid from glucose and maltose • *N. lactamica* (ATCC 23970) produces acid from glucose, maltose, and lactose • *N. mucosa* (ATCC 19696) produces acid from glucose, maltose, and sucrose • *N. cinerea* (ATCC 14685) glucose negative, but may produce a weak glucose reaction; does not produce acid from the other sugars.	
Enzyme substrate test	Use the QC strains recommended by the test manufacturer.* * If the manufacturer has not designated specific strains for QC: • *N. gonorrhoeae* (ATCC 49226) produces hydroxyprolylaminopeptidase. • *N. meningitidis* (ATCC 13077) produces γ-glutamylaminopeptidase. • *N. lactamica* (ATCC 23970) produces ß-galactosidase. • *M. catarrhalis* (ATCC 25238) produces none of these enzymes.	

Note: Laboratorians should follow QC strain designations provided by manufacturers of (commercial) tests; however, if specific strain numbers are not provided, those included in this table can be used for guidance.

Superoxol / Catalase

The superoxol test is a simple test that uses 30% hydrogen peroxide (H_2O_2) as a reagent. Reactions of superoxol with *N. gonorrhoeae* are typically "explosive" (4+, very strong), compared with weaker (2+) reactions with most non-gonococcal *Neisseria* species, and a negative reaction with *K. denitrificans*. In contrast, the catalase test is performed with 3% hydrogen peroxide and yields much weaker results. **This laboratory manual suggests performing the superoxol test (30% H_2O_2) if the reagent is available.** This is because results with the superoxol reagent are more differential for *N. gonorrhoeae* than those obtained with the catalase reagent.

a) Using a sterile inoculating loop or swab, remove some 18–24 hour growth from a pure culture on either selective or non-selective medium, and put it on a clean slide.[20]

b) Using an eye-dropper or a pipette, place a drop of reagent onto the growth.

c) *N. gonorrhoeae* typically has a very strong (4+), "explosive" reaction to contact with superoxol reagent, as pictured in Figure 22. Catalase will give a much weaker (1+ or 2+) reaction.

d) Follow steps *a* and *b* to perform the superoxol/catalase test on positive and negative QC strains. (Examples of QC strains are included in Table 7.)

It should be noted that some strains of *N. meningitidis* and *M. catarrhalis* will have a strong superoxol reaction that is not 'explosive' upon the addition of the hydrogen peroxide but can appear as such to an eye unfamiliar with the characteristic reaction of *N. gonorrhoeae*. This test, therefore, is not definitive for *N. gonorrhoeae*, although it remains differential.

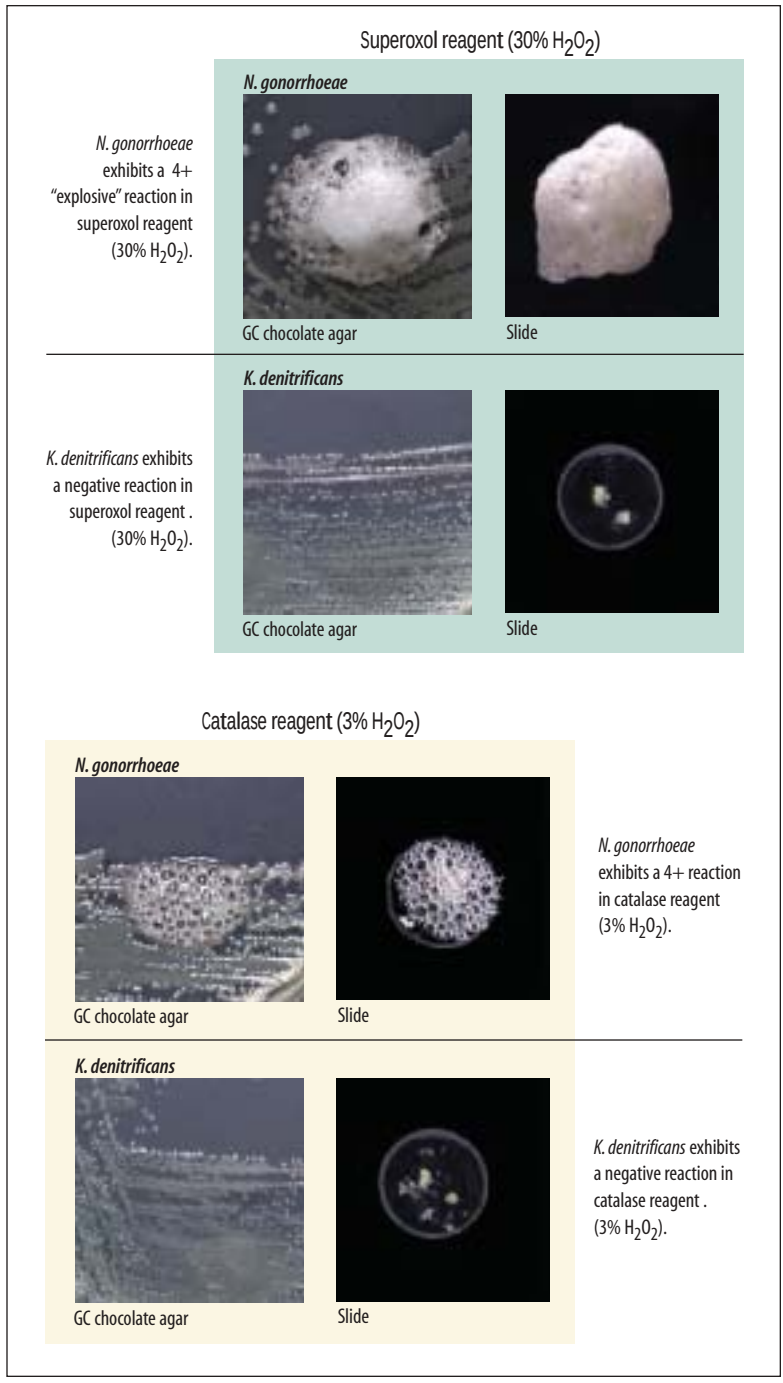
Colistin resistance

Resistance to colistin can be determined either on a selective medium containing colistin (*e.g.*, MTM or ML), or on GC-chocolate agar using the principles of disk diffusion (with a 10 µg colistin disk). A disk diffusion method for qualitative measurement of colistin resistance is presented here.

a) Turn a plate of medium so that it is lid-down on a table. Use a waterproof marker to divide the plate into labeled sectors for the test strain(s), the positive control and the negative control. Examples of QC strains are included in Table 7.

[20] The superoxol / catalase tests can be performed directly on a plate. **However,** it should be noted that hydrogen peroxide reacts with red blood cells, although reactions have not been noted on GC-chocolate agar. If the test is to be performed on an agar plate, place a drop of the reagent on the surface of an uninoculated plate of the medium (or an area of the test plate that does not contain growth) to ensure that no reaction occurs with medium and reagent alone; if a reaction does occur, the test must be performed on a slide (or in a petri dish).

FIGURE 22: Positive and negative reactions in superoxol (30% H_2O_2) and catalase (3% H_2O_2) reagents



- A 100-mm plate can be divided into four sectors, permitting testing of two clinical strains alongside the positive and negative controls. If there are multiple clinical strains requiring the colistin resistance test at once, **and the colistin disks are from the same batch,** it is appropriate to run the positive and negative controls on only one plate.

b) Prepare a suspension of a pure overnight culture (approximately equal to a 0.5 McFarland turbidity standard) in Mueller-Hinton broth or phosphate buffered saline (PBS).

c) Using a sterile swab or inoculating loop, inoculate the GC-chocolate agar plate evenly with a swab. Allow the plate to dry so that there is no visible surface moisture.

d) Apply a colistin disk (10 μg) to the center of the plate, tapping it down to ensure even contact with the surface. Incubate at 35˚–36.5˚C in 5% CO_2 and increased humidity for 18–24 hours.

After incubation, examine the plate for inhibition of growth around the colistin disk. *N. gonorrhoeae* is colistin-resistant, and will grow all the way up to the disk, as will all strains of *N. meningitidis*, *N. lactamica* and *K. denitrificans*. In contrast, strains of commensal *Neisseria* species, most of which are colistin-susceptible, will exhibit zones of inhibition at least 10 mm in diameter with a non-standardized inoculum. Some strains of *N. subflava* biovars, *N. cinerea*, and *M. catarrhalis* may be sufficiently resistant to colistin so as to also grow up to the disk. Thus, the colistin resistance test is not definitive for *N. gonorrhoeae* but will aid in differentiating between this species and many commensal species.

Polysaccharide production test

Some species produce a starch-like polysaccharide when grown on a medium containing sucrose. Upon addition of a drop of Gram's iodine to the growth, this starch will immediately stain dark blue-purple to brown or black. This test is easy to perform and is a useful differential test to be used in combination with others (*e.g.*, superoxol, colistin resistance, acid production) in the identification of *N. gonorrhoeae*. **It is not possible to detect polysaccharide in the sucrose-containing medium of rapid acid-detection tests.** The methods for preparation of the medium appropriate for this test (tryptone-based soy agar [TSA] containing 1% sucrose) can be found in Appendix 2.

a) Turn a plate of sucrose medium so that it is lid-down on a table. Use a waterproof marker to divide the plate into labeled sectors for the test strain(s), the positive control and the negative control. (Examples of QC strains are included in Table 7.)

- A 100-mm plate can be divided into four sectors, permitting testing of two clinical strains alongside the positive and negative controls. If there are multiple clinical strains requiring the polysaccharide test at once, **and the**

plates of medium are from the same batch, it is appropriate to run the positive and negative controls on only one plate.

b) Use a sterile swab or loop to inoculate the polysaccharide test medium with pure culture.
   - Although this test is best performed on isolated colonies, because *N. gonorrhoeae* and strains of some other species do not grow well on this medium, the plate should be inoculated heavily for confluent growth so that the test can detect starch produced by pre-formed enzyme in the inoculum itself.

c) Incubate medium at 35˚–36.5˚C in a CO_2-enriched, humid atmosphere for 18–24 hours.
   - **It is important that this test be performed on growth no more than 24 hours old.** This is because on prolonged incubation the organisms may metabolize the polysaccharide, thus resulting in a false-negative reaction.

d) Use a Pasteur pipette, eyedropper, or inoculating loop to add one drop of Gram's iodine to growth on the plate. Isolates that produce polysaccharide will immediately turn a dark color (brown, purple, black), as shown in Figure 23.
   - If the growth immediately changes color with the addition of Gram's iodine, the strain is considered "polysaccharide-positive." Examples of polysaccharide-positive organisms include *N. polysaccharea*, *N. mucosa*, *N. sicca*, and *N. flavescens.*
   - If the growth does not change color (other than acquiring the light-brown color of the iodine reagent), the reaction is negative, and the strain is considered "polysaccharide-negative." ***N. gonorrhoeae* is polysaccharide-negative,** as are, *e.g.*, *K. denitrificans*, *M. catarrhalis*, *N. cinerea*, *N. lactamica*, and *N. meningitidis.*

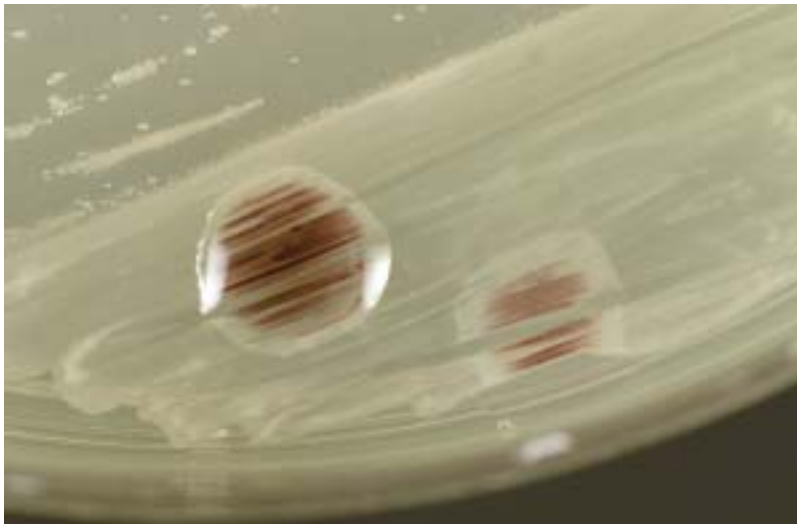
Quality control should be performed with each new batch of sucrose medium or reagent. **This is particularly important because some commercial preparations of Gram's iodine will not react with the starch, yielding false-negative results.** Examples of controls for the polysaccharide-production test are listed in Table 7.

Acid production test

As of the time of writing of this laboratory manual (2002), **it is no longer advised that cystine trypticase agar (CTA) containing glucose, maltose, lactose or sucrose be used for acid production tests for *N. gonorrhoeae.*** The rationale for this shift in procedure is because many strains of *N. gonorrhoeae* produce very little acid from glucose and the color change is not observed in the CTA-sugar media, thus yielding incorrect identifications.

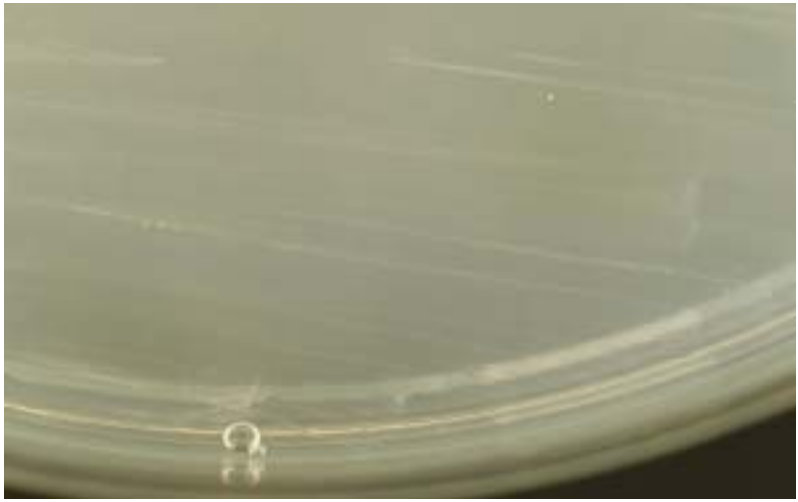
FIGURE 23: Positive and negative results of the polysaccharide-production test on sucrose medium

Polysaccharide postitive *N. polysaccharea*



Organisms able to produce polysaccharide from sucrose turn a brown to blue-black color with the addition of Gram's iodine to growth on sucrose medium and are termed "polysaccharide-positive."

Polysaccharide negative *N. gonorrhoeae*



Organisms unable to produce polysaccharide from sucrose do not undergo a color change with the addition of Gram's iodine to growth on sucrose medium and are termed "polysaccharide-negative."

(***Note:*** polysaccharide-negative colonies may acquire the light brown-yellow color of the iodine reagent.)

Because CTA-sugar media can exhibit misleading results for some strains of *N. gonorrhoeae*, as described above, this laboratory manual advises that, if available, a commercial test be used if it is necessary to detect acid production to confirm the identification of an isolate as *N. gonorrhoeae*. Perform the test according to the manufacturer's instructions and using the manufacturer's recommendations for quality control; note that incubation of the acid production test must occur in an atmosphere **without** supplemental CO_2 in order to avoid false-positive results. It is important that the test chosen to detect acid production be able to differentiate between *N. gonorrhoeae* and *N. cinerea* and *M. catarrhalis*. Reaction patterns of various *Neisseria* species in the acid production test are illustrated in Figure 24.

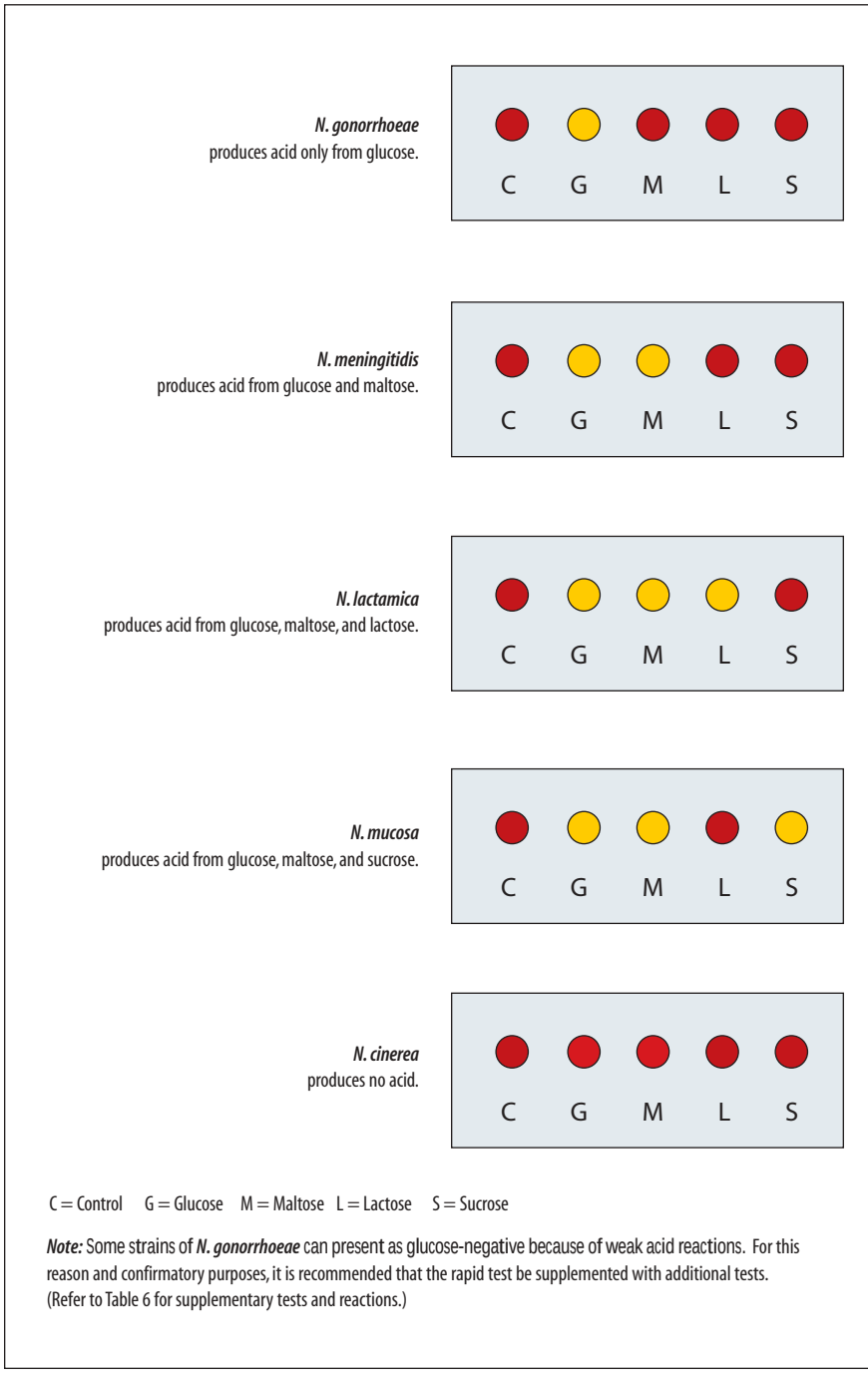
Many of the commercial acid production tests were developed to differentiate among species that routinely grow on selective media for the gonococcus, including *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica* and *M. catarrhalis*. However, interpretive criteria provided with the product (package insert) may not provide guidance for the identification of *K. denitrificans*, *N. subflava* biovars, and *N. cinerea* strains, all of which may also grow on gonococcal selective media. The laboratory will therefore want to ensure that the product can distinguish *N. gonorrhoeae* from these other species, or perform additional tests to allow the correct identification of *N. gonorrhoeae*. Table 6 provides information to guide the choice of supplemental tests for the differentiation of *N. gonorrhoeae* from other species.

It is suggested that a strain of *N. cinerea* be included among QC strains for the acid production test (in addition to *N. gonorrhoeae* and others). Although *N. cinerea* is considered to be glucose-negative and will be listed as such in tables of acid production reactions, it does actually produce acid from glucose and then rapidly over-oxidize it to produce CO_2 and water; as a result, it may either appear negative or give a weak positive glucose reaction (due to residual acid produced from the glucose and not over-oxidized, and/or due to residual carbonic acid from the production of CO_2), and it is therefore useful to compare this reaction to that of the *N. gonorrhoeae* control strain. In addition to *N. cinerea*, follow the quality control instructions provided by the manufacturer of the commercial kit; if the manufacturer has not provided specific QC strain designations, guidance for the selection of appropriate QC strains is provided in Table 7.

Enzyme substrate test

The chromogenic enzyme substrate test detects enzymes (ß-galactosidase, γ-glutamylaminopeptidase, and hydroxyprolylaminopeptidase), and is considered "chromogenic" because color changes indicate the presence or absence of certain enzymes in different *Neisseria* species. The test is commercially available and should be performed according to the manufacturer's directions. (Figure 25 in this laboratory manual shows the Gonochek-II®.) Because most enzyme substrate tests were developed to differentiate only among the organisms believed to grow on media selective for *N. gonorrhoeae*, documentation provided with the product is

FIGURE 24: Acid production commercial test kit results for ***Neisseria gonorrhoeae*** and related organisms



usually limited to distinguishing between *N. gonorrhoeae* (which produces only hydroxyprolylaminopeptidase), *N. meningitidis* (which produces γ-glutamyl-aminopeptidase), *N. lactamica* (which produces ß-galactosidase), and *M. catarrhalis* (which produces none of these three enzymes). It is now known that strains of several commensal *Neisseria* species can grow on selective GC media and also produce only hydroxyprolylaminopeptidase. The chromogenic enzyme substrate test is therefore not definitive for the identification of *N. gonorrhoeae*. Table 6 provides information to guide the choice of supplemental tests for the differentiation of *N. gonorrhoeae* from other species. Follow the quality control instructions provided by the manufacturer of the commercial kit; if the manufacturer has not provided specific QC strain designations, guidance for the selection of appropriate QC strains is provided in Table 7.

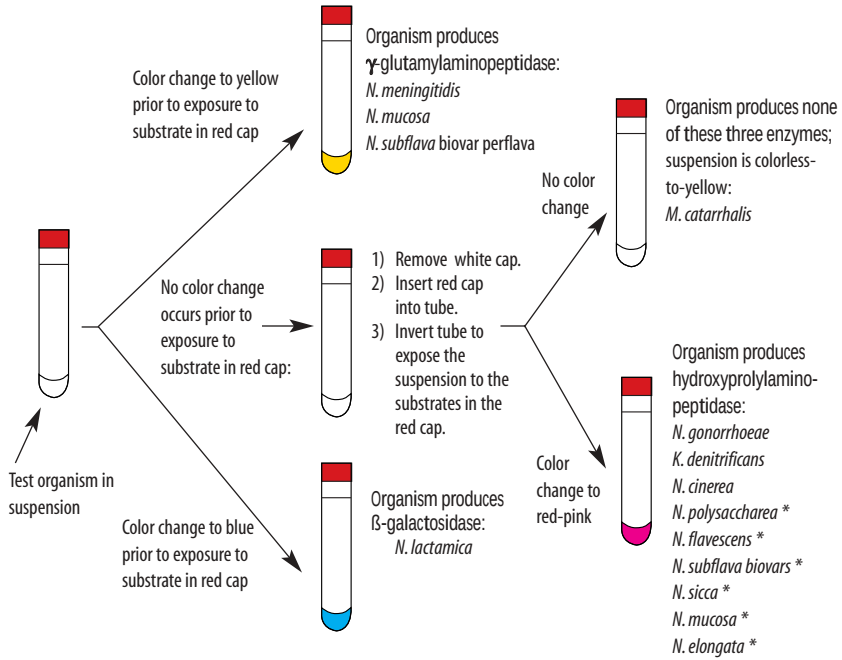
Nitrate reduction test

The nitrate reduction test is available commercially or can be made easily in the laboratory. This test distinguishes between species that can reduce nitrate (NO_3) to nitrite (NO_2) or nitrogenous gases. In the context of this chapter, the test is useful for differentiating between strains of *N. gonorrhoeae* (nitrate-negative) and *K. denitrificans* or *M. catarrhalis* (two nitrate-positive species sometimes misidentified as *N. gonorrhoeae*).

The nitrate reduction test uses a medium containing nitrate and three different reagents: sulfanilic acid ("Nitrate Reagent A"), α-naphthylamine ("Nitrate Reagent B"), and zinc powder ("Zn^{+2} dust"). Bacteria able to reduce nitrate from the medium into either nitrite or into nitrogenous gases are "nitrate-positive," while bacteria that lack enzymes to reduce nitrate are "nitrate-negative."

In practical terms, the nitrate reduction test centers around the colorimetric detection of nitrite in the test medium. Nitrite forms a compound with sulfanilic acid, which when reacted with α-naphthylamine gives a pink-to-red color depending upon the concentration of nitrite in the medium; the addition of Nitrate Reagents A and B is therefore only able to detect the presence of nitrite in the medium. If a pink-red color is detected after the addition of Nitrate Reagents A and B, the organism is considered to be "nitrate-positive." However, if there is no color change in the medium after the addition of these reagents, it is necessary to determine whether nitrate was ever reduced to nitrite, or whether the nitrite produced was completely reduced to nitrogenous gases. This is accomplished by using a small amount of zinc powder, which chemically catalyzes the reduction of nitrate to nitrite and nitrite to nitrogenous gases. (It is therefore critical to use only a very small amount of zinc powder so that if nitrate has not been reduced by enzymes produced by the bacteria, the reaction catalyzed by the zinc powder is not so strong as to reduce the nitrate completely to nitrogenous gases so rapidly that it is not possible to detect the nitrite produced in the catalytic reaction in the medium.) **Nitrate-negative strains will exhibit a color change to red after**

FIGURE 25: Reactions of ***Neisseria gonorrhoeae*** and related organisms in a commercial enzyme substrate test



The enzyme substrate test is a chromogenic test used to identify organisms that produce γ-glutamylaminopeptidase (indicated in this example of commercial test kit results as a color change to yellow), ß-galactosidase (indicated in this example as a color change to blue), hydroxyprolylaminopeptidase (indicated in this example as a color change to red-pink), and none of these enzymes (indicated by an absence of change of color in the cell suspension in the tube).

(* Species which do not usually grow on selective media for *N. gonorrhoeae* but may do so occasionally and give a positive hydroxyprolylaminopeptidase reaction in an enzyme substrate test.)

incubation with zinc powder (nitrate is reduced to nitrite by the zinc powder, and the nitrite is detected by Nitrate Reagents A and B already in the medium, yielding a color change to pink-red). Nitrate-positive strains do not exhibit a color change after incubation with zinc powder because nitrate in the medium will have already been reduced beyond nitrite to nitrogenous gases. To summarize:

- Bacteria that reduce nitrate to nitrite may be identified when addition of Nitrate Reagents A and B causes the medium to change color from clear to pink-red; no additional testing with zinc powder is required. Results should be recorded as "nitrate-positive."
- Bacteria that reduce nitrate to nitrite and then further reduce the nitrite to nitrogenous gases are identified when there is no color change in the medium after either the addition of Nitrate Reagents A and B, or after incubation with zinc powder. Results should be recorded as "nitrate-positive."

- Bacteria unable to reduce nitrate at all are identified when there is no color change with the addition of Nitrate Reagents A and B, but there **is** a color change in the medium from clear to pink-red after incubation with zinc powder. Results should be recorded as "nitrate-negative."

The nitrate test is performed in a standard nitrate broth which is inoculated heavily to give a dense suspension of organisms because many *Neisseria* species may not grow in this medium; the reaction for these species will therefore depend upon preformed enzymes in the inoculum. The test must be performed exactly as described; if not performed correctly, the test results may be inaccurate and an incorrect identification made. A schematic representation of the nitrate reduction test is shown in Figure 26. Media and reagents required for this test are described in Appendix 2.

Nitrate reduction occurs only under anaerobic conditions; it is therefore important to ensure a low surface-area to depth ratio to limit the diffusion of oxygen into the medium during the test. These conditions will be met by dispensing 5 ml of medium into a 13 mm diameter screw-cap tube.

It is important to run a medium control and both negative- and positive- controls as the test is complex and the controls have known outcomes to indicate if the media and reagents are reacting appropriately. **Quality control tests should be performed each time clinical isolates are tested,** using QC strains included in Table 7.

Methods

a) Using colonies from a fresh, pure culture on GC-chocolate agar, prepare a heavy suspension in nitrate broth.

b) Remove the screw-cap top from the tube of nitrate test medium and inoculate the medium to give heavy turbidity. Replace the screw-cap top.

c) Incubate the inoculated tubes and an uninoculated medium control tube at 35˚–36.5˚C (without supplemental CO_2) for 48 hours.

d) After incubation for 48 hours, remove the screw-cap top from the tube. Add 5 drops of Nitrate Reagent A to each tube (including the uninoculated control medium). Shake each tube gently back and forth to mix Reagent A with the medium, add 5 drops of Nitrate Reagent B to each tube (again including the uninoculated control medium), and again shake each tube gently back and forth to mix Reagent B with the medium.
   - **If the uninoculated control medium turns pink-red,** the test is invalid, and a new batch of media must be prepared.
   - **If the uninoculated control medium shows no color change,** proceed to step *e*.

e) Examine the test medium and controls for a pink-red color; this color should develop within a few minutes if the medium is still warm. The reaction may take a little longer if the medium has cooled before the reagents are added.
   - The negative control medium should show no color change.
   - The positive control medium may or may not exhibit a color change to pink-red, depending upon whether nitrate was reduced to nitrite or further reduced to nitrogenous gases.
   - **If the test medium turns pink-red after the addition of Nitrate Reagents A and B,** the reaction is positive and the test is completed. If a pink-red color develops, do not perform step *f* and **record the reaction as nitrate-positive.**

f) If the medium is still colorless after the addition of Nitrate Reagents A and B, add a very small amount of zinc powder to the medium. (A convenient method to estimate the amount of zinc powder required for the test is to use the sharp point of a knife to pick up the powder; the pile of zinc powder should not exceed 4–5 mg, or 2–3 mm in diameter.) Shake the tube vigorously back and forth to mix well, and allow it to stand at room temperature for 10–15 minutes.
   - If the negative control turns pink-red after the addition of zinc powder, the amount of zinc added is sufficient for the reaction to occur (and not so much as to cause rapid over-reduction of nitrate to nitrogenous gases). Continue by interpreting the reactions in the test media.
   - **If the medium remains colorless after the addition of zinc powder,** the test result is positive (nitrate has been reduced to nitrite and further reduced to nitrogenous gases). Record the result for the isolate as "nitrate-positive."
   - **If the medium turns pink-red after the addition of zinc powder,** the result is negative. Record the result for the isolate as "nitrate-negative." *N. gonorrhoeae* is nitrate-negative.

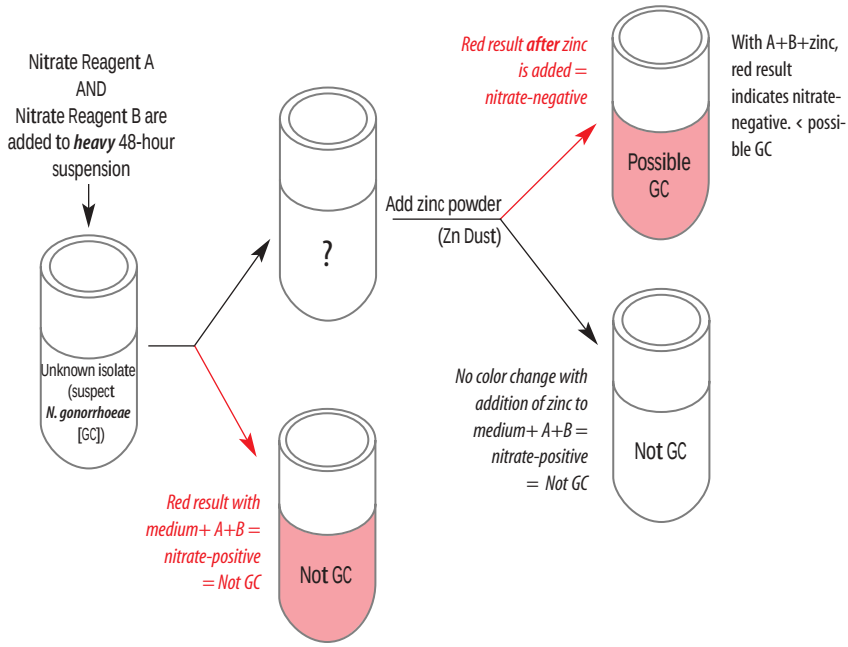
No identification of genus or species can be made on the basis of any of the above biochemical and enzymatic tests alone, but performing a combination (*e.g.*, as presented in Figure 21) can lead to a definitive identification of *N. gonorrhoeae.*

Antimicrobial susceptibility testing of *N. gonorrhoeae*

The methods presented in this laboratory manual are those recommended by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),[21] although a variety of methods are used internationally to determine antimicrobial susceptibilities of *N. gonorrhoeae.*

[21] Formerly known as the "National Committee for Clinical Laboratory Standards," NCCLS is now known solely by its acronym.

FIGURE 26: Schematic representation of the nitrate reduction test



Note: Each nitrate reduction test must have three controls: one positive, one negative, and one just medium. As a result, four complete tests will need to be performed to interpret the result for one test isolate. The medium control and negative control should each always yield a negative reaction; a positive control should always yield a positive reaction. If these results do not occur, start the test over with a new suspension, new media, and new reagents.

N. gonorrhoeae is "nitrate-negative," so if the pathway shows a nitrate-positive result and the controls are functioning properly, the isolate is not a gonococcus.

These methodologies are currently (2002) being reviewed by the International Collaboration on Gonococci (ICG), and it is possible that some modifications will be made to the methods described in this document.[22]

Minimal inhibitory concentration (MIC) determination by the agar dilution method is the reference method ("gold standard") for determining the antimicrobial susceptibilities of *N. gonorrhoeae* isolates. However, this method is complex to perform, and so is beyond the scope of this manual.[23] Antimicrobial susceptibilities can also be determined by the disk diffusion test, or MICs can be obtained with the Etest® (AB Biodisk). This document presents the methods for antimicrobial susceptibility testing of *N. gonorrhoeae* with the antimicrobial agents

[22] International gonorrhea reference laboratories can provide additional information on ICG activities; contact information for these laboratories is included in Appendix 14.

[23] Laboratorians interested in learning more about agar dilution antimicrobial susceptibility test methods may contact an ICG reference laboratory (Appendix 14).

currently recommended by WHO for the primary therapy of gonorrhea: ciprofloxacin, azithromycin, ceftriaxone, cefixime, and spectinomycin [WHO 2001].

Factors such as testing medium, inoculum size, incubation atmosphere, and antimicrobial disk concentrations may affect the antimicrobial susceptibility values obtained. Thus, quality control is of great importance and, with every test run, laboratory personnel must include reference strains with known antimicrobial susceptibilities to ensure that the susceptibility results for test isolates are accurate. It should be noted that for methods that determine MICs, the MIC will be accurate plus or minus (±) one dilution. For example, an organism with an MIC of penicillin of 0.25 μg/ml may exhibit an MIC of 0.125 μg/ml to 0.5 μg/ml, but it would be found upon repeated testing that most antimicrobial susceptibility values (*i.e.*, the modal MIC) for this organism and drug would be 0.25 μg/ml. Disk diffusion results (*i.e.*, inhibition zone diameters, mm) exhibit a similar normal distribution upon repeated testing of the same isolates. It is important to keep these variations of measurement in mind as laboratories typically perform only one complete set of antimicrobial susceptibility tests per isolate, and not repeated measures for the same antimicrobial agent unless there is a specific reason to do so, such as confirming an unusual antimicrobial susceptibility result.

WHO has recommended a number of isolates for quality control (QC), although these do not adequately represent the variety of resistance patterns now known to exist for *N. gonorrhoeae*. Consequently, most international laboratories have included additional QC strains exhibiting resistance and intermediate resistance to fluoroquinolones and emerging resistance to azithromycin. Only one strain, *N. gonorrhoeae* ATCC 49226, is designated by NCCLS for QC of antimicrobial susceptibility testing of gonococcal isolates. At the Centers for Disease Control and Prevention (CDC), the NCCLS-recommended QC strain and supplemental QC strains are routinely made available to investigators (see Appendix 14 for contact information). Strains of *N. gonorrhoeae* are currently being tested under ICG sponsorship to establish an international reference panel for QC of antimicrobial susceptibility testing that represents the known range of resistances in this species.

Once the susceptibility of a gonococcal strain to an antimicrobial agent has been measured *in vitro*, the strain is then classified as susceptible, intermediate, or resistant to each antimicrobial agent tested to indicate whether the infection may either respond fail to respond to therapy with that agent. For clinical applications (*i.e.*, prescribing appropriate therapy for individual patients), antimicrobial susceptibilities are always interpreted strictly according to standardized guidelines, such as the NCCLS interpretive criteria. These criteria must be specific for the dose of the agent used to treat the infection [Knapp *et al.* 1995]. For example, NCCLS criteria for the interpretation of susceptibility of *N. gonorrhoeae* to ciprofloxacin were developed to correspond to treatment with the recommended 500-mg of ciprofloxacin in a single oral dose; assessment of treatment efficacy of a single oral dose of 250-mg of ciprofloxacin would require the development of separate interpretive criteria.

Organism-antimicrobial-dose interactions are categorized into one of two levels of classification based on the clinical efficacy of the antimicrobial agent. One level applies to antimicrobial agents to which an organism has not yet developed clinically significant resistance, and uses the categories "susceptible" and "decreased susceptibility." The second level is used when the organism has developed clinically significant resistance resulting in failure of the infection to respond to therapy with the recommended dose of the antimicrobial agent ("treatment failures"), and uses the categories "susceptible", "intermediate," and "resistant." For example:

- At the time of writing (2002), gonococcal infections have not been confirmed to fail to respond to therapy with extended-spectrum cephalosporins, such as cefixime (400-mg in a single oral dose). The NCCLS has established an interpretive criterion of "susceptible" as an MIC of ≤ 0.25 mg/ml of cefixime (corresponding disk diffusion zone of inhibition diameter with a 5-μg cefixime disk, ≥ 31mm). Organisms with a higher MIC (or smaller inhibition zone diameter) are classified as exhibiting "decreased susceptibility" to cefixime.
- When infections fail to respond to recommended therapies with specific antimicrobial agents, a "resistant" category is established for that organism-antimicrobial-dose combination by NCCLS. Breakpoints are set for *in vitro* determination of this category based on testing of a variety of isolates resistant to the recommended therapeutic treatment. For example, gonococcal infections caused by organisms for which the ciprofloxacin MICs are ≥ 1.0 mg/ml (corresponding disk diffusion zone diameter of inhibition with a 5 mg ciprofloxacin disk, ≤ 27mm) have failed to respond to therapy with the WHO-recommended single oral ciprofloxacin dose of 500-mg. NCCLS had previously defined the "susceptible" breakpoint for ciprofloxacin as an MIC of ≤ 0.06 mg/ml (zone inhibition diameter ≥ 41 mm), so the "intermediate" designation applies to those isolates for which the MICs are in the range between the susceptible and resistant categories, *i.e.,* 0.125 μg/ml – 0.5 μg/ml (28 mm – 40 mm). For gonococcal infections, it should be noted that organisms in the "intermediate" category for an antimicrobial agent have rarely been associated with confirmed instances of treatment failure with that agent.

NCCLS interpretive criteria are designed to define antimicrobial susceptibility test result categories when NCCLS methodology is used to perform the tests, as presented in this laboratory manual.[24] The additional QC reference strains included in this laboratory manual for antimicrobial agents not currently included in NCCLS guidelines have been validated by the Gonorrhea Research Branch (*Neisseria* Reference Laboratory) at the CDC, and may be used alongside NCCLS criteria with the methods presented here until an ICG-sponsored international QC

[24] NCCLS methods are presented in this document, and are strongly recommended. However, if a laboratory uses different antimicrobial susceptibility testing methodologies for *N. gonorrhoeae*, and all quality control references are consistently in check with the NCCLS interpretive criteria for QC strain ATCC 49226, the laboratory may consider interpreting results for the alternate testing methodology according to the NCCLS interpretive criteria.

reference panel is designated. Tables 9 and 10 provide summaries of QC ranges and interpretive criteria for clinical isolates.[25]

In resource-limited geographic areas or in local clinical laboratories, **antimicrobial susceptibility test results should be determined for current antimicrobial therapies and also the alternate antimicrobial agent(s) that would be used if resistance emerged to the current regimen.** Not all local laboratories will have the capacity to perform antimicrobial susceptibility testing on isolates. National or large regional laboratories acting in the capacity of a reference laboratory should be able not only to provide assistance to local laboratories and health authorities (clinical applications), but also to perform the most extensive susceptibility testing to a broad range of antimicrobial agents in order to compare susceptibilities of isolates at the regional, national and international levels (surveillance activities).[26] In a local laboratory, if it is not feasible to perform prospective surveillance, the laboratory should at least determine susceptibilities of post-treatment "treatment failure" isolates which could either be truly resistant treatment failures or else susceptible isolates acquired by re-infection. If a laboratory is unable to perform antimicrobial susceptibility testing, isolates should be sent to a laboratory that can perform such testing. (Methods for preservation and storage of isolates are included in Appendix 11; transport of isolates is addressed in Appendix 12.)

In addition to providing immediate assistance to local and regional laboratories and public health authorities in efforts to control gonorrhea by determining antimicrobial susceptibilities to the recommended therapies, reference laboratories may want to conduct more extensive antimicrobial susceptibility testing in order to develop a global perspective on antimicrobial resistance in *N. gonorrhoeae.* Determination of antimicrobial susceptibilities to a wide range of agents—penicillin, tetracycline, spectinomycin, extended-spectrum cephalosporins (*e.g.*, ceftriaxone and cefixime), fluoroquinolones (*e.g.*, ciprofloxacin, ofloxacin, and levofloxacin), and the macrolide azithromycin—allows for the comparison of isolates from the population served by the testing laboratory with isolates from other regions.

Laboratory-based surveillance for antimicrobial resistance may be conducted at one of two basic levels. When resources are limited, surveillance may be performed for susceptibilities to antimicrobial agents being used for primary and secondary therapy of gonorrhea, *i.e.*, the primary agent being used to treat infections and the alternative therapeutic agent(s) that would be used to treat infections not treated effectively by the primary regimen. In this instance, antimicrobial susceptibilities

[25] If antimicrobial susceptibility test QC results for a locally developed testing method are consistent but do not agree with those obtained by NCCLS-recommended methods, the testing laboratory may want to consult with the ICG for assistance with the development of standard interpretive criteria appropriate to the situation.

[26] Laboratorians interested in learning more about the methods used for the surveillance of antimicrobial resistance in *N. gonorrhoeae* isolates can find links to various protocols through the following internet address: **http://www.cdc.gov/ncidod/dastlr/gcdir/gono.html**

would be interpreted by the standards used for clinical applications, *e.g.*, by NCCLS standards.

When investigators wish to compare the antimicrobial susceptibilities of *N. gonorrhoeae* strains in their geographic locality with those in other geographic areas, susceptibilities are usually determined to a larger number of antimicrobial agents than those used locally for treatment. A typical panel might include the following: penicillin, tetracycline, spectinomycin, an extended-spectrum cephalosporin (*e.g.*, ceftriaxone or cefixime), a fluoroquinolone (*e.g.*, ciprofloxacin, ofloxacin, or levofloxacin), and a macrolide (*e.g.*, azithromycin). For broad surveillance purposes, gonococcal isolates are described first by their susceptibilities to penicillin and tetracycline (although these drugs should not be used treat gonorrhea) and by a simple test to detect the production of ß-lactamase (described below). This is because, based on the level of resistance to penicillin and tetracycline and the detection of ß-lactamase, it is possible to predict whether the mechanisms of resistance to penicillin and tetracycline are chromosomally mediated or plasmid-mediated.

A specialized classification and terminology with standard acronyms has been developed to describe patterns of penicillin-tetracycline resistance and designate penicillin-tetracycline resistance phenotypes, as presented in Table 8. Organisms that are ß-lactamase-negative and resistant to penicillin but not tetracycline, for example, use the NCCLS designation "penicillin-resistant," and are designated "PenR." Other acronyms do not use NCCLS designations in their names, although NCCLS methods are used to identify the resistances. For example, "CMRNG" (chromosomally mediated resistant *N. gonorrhoeae*) describes organisms that have chromosomally mediated resistance to **both** penicillin (MIC ≥ 2.0 mg/ml, or equivalent inhibition zone diameter ≤ 26 mm) **and** tetracycline (MIC ≥ 2.0 mg/ml, or equivalent inhibition zone diameter ≤ 30 mm) **and** do not produce ß-lactamase. It should be noted that while plasmid-mediated resistance to penicillin can be detected and confirmed with a simple test to detect ß-lactamase, plasmid-mediated resistance to tetracycline can only be identified presumptively with susceptibility results and must be confirmed with a complex test demonstrating the presence of the TetM-conjugative plasmid (*e.g.*, by laboratories performing molecular epidemiologic testing).

The basic penicillin-tetracycline resistance phenotype acronym characterizes susceptibilities only of penicillin and tetracycline. For other therapeutic agents, NCCLS (or equivalent) standardized criteria are used to classify susceptibilities to these agents, and antimicrobial resistance (including "intermediate" or "decreased susceptibility" categories) to these additional antimicrobial agents is appended to the penicillin-tetracycline resistance phenotype. For example, a CMRNG isolate exhibiting resistance to ciprofloxacin (CipR) would be cited as "CMRNG, CipR." Such descriptive designations permit one to rapidly appreciate the fact that ciprofloxacin resistance is occurring in an organism already resistant to penicillin

TABLE 8: Phenotypic designations of ***Neisseria gonorrhoeae***

Phenotype	Phenotype definition	ß-lactamase results and specific MIC values associated with phenotype definitions for ***Neisserea gonorrhoeae*** [a, b]
Susceptible	Isolates exhibiting *either* susceptibility *or* intermediate resistance to *both* penicillin and tetracycline	ß-lactamase negative isolate exhibiting: • Susceptibility to penicillin [MIC < 2.0 µg/ml (>26 mm)] • Susceptibility to tetracycline [MIC < 2.0 µg/ml (>30 mm)]
PPNG	Penicillinase-producing *Neisseria gonorrhoeae*	ß-lactamase positive isolate. Approximately six ß-lactamase plasmids have been identified in *N. gonorrhoeae,* most commonly: • "Asian" = 4.4 megadaltons (Mda) (7.2 kb) • "African" = 3.2 Mda (5.3 kb) • "Toronto" = 3.05 Mda (4.7 kb) (PPNG is defined only by production of ß-lactamase and not by MICs of penicillin.) [c]
TRNG	Tetracycline resistant *Neisseria gonorrhoeae*	ß-lactamase negative isolates possessing a TetM-containing conjugative plasmid. TRNG isolates will exhibit both: • Susceptibility to penicillin [MIC < 2.0 µg/ml (>26 mm)] • Resistance to tetracycline with MIC ≥ 16.0 µg/ml (≤20 mm) Presumptive identification of this phenotype is based on MICs of penicillin and tetracycline. Confirmatory identification of TRNG (TetM and subtyping) is by PCR
PP/TR	Penicillinase-producing, tetracycline resistant *Neisseria gonorrhoeae*	ß-lactamase positive isolates of *N. gonorrhoeae* exhibiting: • Resistance to tetracycline with MIC ≥ 16.0 µg/ml (<20 mm)
PenR	Chromosomally mediated resistance to penicillin	ß-lactamase negative isolates exhibiting both: • Resistance to penicillin with MIC ≥ 2.0 µg/ml (≤26 mm) • Susceptibility to tetracycline [MIC < 2.0 µg/ml (>30 mm)]
TetR	Chromosomally mediated resistance to tetracycline	ß-lactamase negative isolates exhibiting both: • Susceptibility to penicillin [MIC < 2.0 µg/ml (>26 mm)] • Resistance to tetracycline with an MIC range of 2.0 µg/ml - 8.0 µg/ml (20 – 30mm)
CMRNG	Chromosomally mediated resistant *Neisseria gonorrhoeae*	ß-lactamase negative isolates exhibiting both: • Resistance to penicillin with MIC ≥ 2.0 µg/ml (≤26 mm) • Resistance to tetracycline with MIC ≥ 2.0 µg/ml (≤30 mm)

[a] ***Note:*** Some TRNG may exhibit tetracycline MICs <16.0 µg/ml, and some TetR isolates may exhibit tetracycline MICs ≥16.0 µg/ml. The difference between TRNG and TetR can only be confirmed by a test to determine the presence or absence of the TetM plasmid.

[b] ***Note:*** For some research purposes, a breakpoint of 1.0 µg/ml of penicillin and tetracycline is used to differentiate more equitably between (penicillin and tetracycline) susceptible isolates and isolates belonging to the CMRNG group of organisms [Rice and Knapp 1994].

[c] ***Note:*** For PPNG isolates, MICs for penicillin are typically high (≥8.0 µg/ml) (≤20 mm); however, it is possible for them to be lower and have larger zones of inhibition as well. Some PPNG isolates have MICs as low as 0.25 µg/ml of penicillin but are still ß-lactamase positive.

and tetracycline. The use of penicillin-tetracycline resistance phenotypes also has practical applications for monitoring susceptibilities to the extended-spectrum cephalosporins: gonococcal isolates exhibiting chromosomally mediated, high levels of resistance to penicillin (PenR) or penicillin and tetracycline (CMRNG) exhibit higher—but still susceptible—MICs of ceftriaxone and cefixime.

Aggregation and analysis of phenotypic data permit investigators to monitor changes in the prevalence of resistant strain populations and their geographic patterns of spread, and these surveillance tools may be used to help anticipate the need to revise treatment recommendations before resistance becomes endemic in a region and undermines the effectiveness of local gonorrhea control measures.

Further characterization of resistant strains

An area of research in which reference laboratories may be interested in participating is the further subtype characterization[27] of isolates exhibiting the same antimicrobial resistance phenotypes. Subtyping methods are resource-intensive, however, and so it is not expected that every reference laboratory will be able to adopt these techniques. Genotypic and phenotypic subtyping characterizes individual strains and facilitates a refined interpretation of the antimicrobial resistance data. By assigning strain subtype designations, investigators may be able to differentiate between the strain types which are sporadically imported and coincidentally exhibit the same resistance phenotype as a local strain. Strain subtyping coupled with information about social-sexual networks may facilitate proactive disease control interventions.

Methods for detecting antimicrobial resistance in *N. gonorrhoeae*

As detailed above, there are two different approaches taken when defining for what antimicrobial agents susceptibility tests should be performed. When performing antimicrobial susceptibility testing for clinical purposes, susceptibilities should be determined to the antimicrobial agents currently used for treatment of gonorrhea and the alternate antimicrobial agent(s) that would be prescribed if the primary course were to be ineffective. When performing antimicrobial susceptibility testing for surveillance purposes, however, the clinical testing is supplemented with an expanded panel of antimicrobial agents in conjunction with ß-lactamase testing, providing the laboratory with phenotypic data appropriate for international comparisons.

Tests identifying gonococcal strains that produce ß-lactamase are used in conjunction with MICs as an integral component of surveillance to differentiate between chromosomally mediated and plasmid-mediated resistance to penicillin for

[27] Examples of phenotypic typing include auxotyping (determination of nutrients required for growth of a strain), serotyping, ß-lactamase plasmid typing, and TetM plasmid typing. Examples of genotypic typing include Lip subtyping, RFLP-related typing, and Opa typing.

N. gonorrhoeae, as explained above. The nitrocefin test is a qualitative test used to detect production of ß-lactamase; it can be performed using the same culture on GC-chocolate agar used to prepare the inoculum for MIC (or disk diffusion) tests.

***Test for ß-lactamase production by* N. gonorrhoeae**

The most reliable way to detect ß-lactamase-producing strains of *N. gonorrhoeae* is to use the nitrocefin test. Reactions are strongest when the test is performed on cultures recently removed from an incubator and still warm. The nitrocefin test is performed either with a liquid reagent or with a treated disk. Because the liquid reagent can be expensive, the disk method is preferable if relatively few isolates are to be tested. Positive and negative controls should be run each time this test is performed. Positive and negative control strains may be selected from among those listed in Table 7.

Nitrocefin disk method

a) Use sterile forceps or tweezers to place a nitrocefin disk on a clean slide.

b) Add a drop of distilled water to the disk and allow it to absorb so the disk is moistened, but not wet.

c) Touch a sterile swab or loop to a characteristic colony in fresh, pure, 18–24 hour culture.

d) Rub the swab on the moistened disk so that the growth goes into the filter paper of the disk.

e) Examine the disk: if the reaction is positive, the areas of the disk containing growth will turn a characteristic red/pink color. Reactions typically occur within five minutes.

f) Record results. Strains for which the inoculum on the nitrocefin disk turns red/pink are considered "ß-lactamase positive". Strains for which the inoculum on the nitrocefin disk does not change color are considered "ß-lactamase negative."

Nitrocefin liquid reagent

If it is anticipated that a large number of isolates will be tested, laboratorians should investigate obtaining nitrocefin powder and preparing the liquid reagent. The nitrocefin test using liquid reagent is performed either by dropping reagent directly on colonies growing on selective or nonselective media, or by diluting the reagent and using it as a suspension medium for bacterial growth in a tube. Although the former method is easier as it involves fewer steps, **the advantage of the latter method is that it uses lesser amounts of the costly liquid reagent**. (Methods for the different preparations of the nitrocefin reagent as used for each of these tests are included in Appendix 2.)

To perform the test for ß-lactamase production with liquid nitrocefin reagent using the plate method, use an eyedropper, Pasteur pipette or inoculating loop to

place a drop of the undiluted reagent directly onto fresh gonococcal colonies growing on selective or nonselective culture media. After several minutes, the colonies will turn pink if the gonococcal strain is producing ß-lactamase, and should be recorded as "ß-lactamase positive." If, after ten minutes, no color change has occurred on the colonies dropped with reagent, the gonococcal strain is considered "ß-lactamase negative," and should be recorded as such.

To perform the test for ß-lactamase production with liquid nitrocefin reagent using the tube method, dispense dilute nitrocefin solution (25 mg/L prepared in 0.1M phosphate buffer) into a test tube, and use it to prepare a heavy suspension (~ McFarland 2) of the suspect gonococcal colonies from 18–24 hour culture. If ß-lactamase producing organisms are present, the suspension should change color from colorless/yellow to pink within 15 seconds; record a strain exhibiting this color change as 'ß-lactamase positive.' If after five minutes no color change has occurred in the suspension, record the strain as 'ß-lactamase negative.'

Results of ß-lactamase tests are used in conjunction with results of antimicrobial susceptibility tests performed according to NCCLS methodologies.

Antimicrobial susceptibility testing of *N. gonorrhoeae* using NCCLS methodologies

Antimicrobial susceptibility testing by both disk diffusion and the antimicrobial gradient strip Etest® method are conducted on the same standardized medium. Because gonococci are fastidious, antimicrobial susceptibility tests for most antimicrobial agents are performed on a GC agar base medium supplemented with IsoVitaleX or an equivalent supplement. **Mueller-Hinton medium, on which susceptibilities of most aerobic bacteria are determined, is not suitable for determining gonococcal susceptibilities;** however, Mueller-Hinton broth can be used to prepare the gonococcal cell suspensions that will be tested. Furthermore, gonococcal susceptibilities should not be determined on media containing chocolatized blood or hemoglobin because of the variability of blood products (which may affect susceptibilities of *N. gonorrhoeae* to various antimicrobial agents). Antimicrobial susceptibility test results for *N. gonorrhoeae* should only be interpreted when tested on GC-susceptibility test medium, a standard quality controlled GC agar base medium plus 1% defined supplement.

A sample form for recording the results of antimicrobial susceptibility tests for *N. gonorrhoeae* is included in Figure 27.

Antimicrobial susceptibility testing of N. gonorrhoeae *by disk diffusion*

Disk diffusion testing should be carried out as defined by the NCCLS performance standards and with the NCCLS quality control strain *N. gonorrhoeae* ATCC 49226. It is recommended that laboratories obtain additional gonococcal reference strains exhibiting resistance patterns not exhibited by ATCC 49226: supplemental QC strains, tested routinely by disk diffusion and agar dilution methods with

FIGURE 27: Sample form for recording antimicrobial susceptibility test results for *Neisseria gonorrhoeae*

Date of Testing: _____/_____/_____
*Test performed by:*_____

Interpretation of susceptibility: ***S*** = susceptible ***I*** = intermediate ***R*** = resistant

Specimen number	*Antimicrobial:* **Ciprofloxacin**	*Antimicrobial:*	*Antimicrobial:*	*Antimicrobial:*	*Antimicrobial:*	*Antimicrobial:*	*Antimicrobial:*	*Antimicrobial:*
	S I R	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*
	S I R	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*
	S I R	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*
	S I R	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*	*S I R*
NCCLS QC strain ATCC 49226 *QC in range?* →	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*
(Other QC strain)[a] *QC in range?* →	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*	*Yes No*

[a] Choice of supplemental QC strains will depend upon the antimicrobial agents tested, and therefore several QC strains may be indicated.

Reveiwed by: _____ ***Date of Report:*** ____/____/_____

Note: After 20-24 hours of incubation, check the results for the quality control ("QC") strains against the acceptable range of inhibition zone diameters (mm) or MICs (µg/ml); if they are in control, continue reading results for the test isolate. (*Inhibition zone ranges and breakpoints for interpretation of results may be found in Tables 9 and 10.*)

consistent results, may be obtained from the *Neisseria* Reference Laboratory, Gonorrhea Research Branch, CDC (see Appendix 14). Quality control values for disk diffusion zone diameter sizes for these strains are presented in Table 9.

Methods

a) Label one plate of GC-chocolate agar for each clinical isolate and QC strain to be tested.

b) Inoculate plates with each test strain and streak for isolation. Incubate inoculated plates at 35°–36.5°C in a CO_2-supplemented atmosphere with increased humidity for 16–20 hours.
   - ***Note:*** if isolates are maintained in culture prior to inoculation for antimicrobial susceptibility testing, they must be subcultured every 24 hours prior to being tested.
   - ***Note:*** if isolates are stored frozen prior to inoculation for antimicrobial susceptibility testing, they must be subcultured at least once after initial culture from the frozen preparation prior to being tested.

c) Suspend isolated colonies (from the overnight cultures prepared in steps a and b) in 1.0–2.0 ml of Mueller-Hinton broth (or PBS). Mix the suspension thoroughly on a vortex mixer to break up clumps of growth as much as possible.
   - It is easier to prepare the suspensions with a swab[28] than with an inoculating loop. The best method to avoid excessive clumping of growth in the suspension is to roll the swab over the colonies rather than to use a scraping method to harvest cells.

d) Adjust the turbidity of the cell suspension to the turbidity of a 0.5 McFarland standard by comparing tubes against black and white lines and adding broth or culture as needed (see Figures 51and 52 in Appendix 2). **The suspension must be used to inoculate the plate within 15–20 minutes after preparation, or else it must be discarded** and a new suspension prepared.
   - ***Note:*** The inoculation step must be completed within 15–20 minutes because the organisms will begin to die within a short time after the suspension is prepared, and even though the suspension will be visually comparable to the McFarland standard, the viability of the inoculum delivered onto the test medium may be too low to produce reliable antimicrobial susceptibility test results.
   - If there are many cultures to test, they should be done in small batches (*e.g.*, five or six isolates at a time) to avoid loss of viability.

[28] Notes on the survival of *N. gonorrhoeae* with different swab materials are included in Table 29, Appendix 8.

e) Pour 60 ml of GC base medium containing 1% defined supplement into a 150-mm diameter plate to a uniform depth of 3–4 mm (in order to assure proper conditions for disk diffusion results). The number of plates required for the testing of each strain will be dependent upon the number and type of antimicrobial agents to be tested, as some have larger inhibition zone sizes than others and the zones of inhibition must not overlap. Generally speaking, GC susceptibility tests have no more than 3 disks on each plate.

   Plates to be used for antimicrobial susceptibility testing must have warmed to room temperature before they are inoculated with the cell suspension. **The surface of the plate must also be dry prior to inoculation;** if not, invert the plates and dry them with lids slightly open either in an incubator at 35˚–36.5˚C, or in a biohazard hood. There should be no visible drops of moisture on the surface of the agar when the plates are inoculated.

f) Moisten a sterile applicator swab[28] in the standardized cell suspension and remove any excess moisture by rotating the swab against the glass above the liquid in the tube. Inoculate the entire surface of each plate three times, rotating the plate 60˚ each time to ensure confluent growth (Figure 34).

g) Store the inoculated plates at room temperature for 3–5 minutes to allow the medium to absorb the moisture from the inoculum. **It is essential that the surface of the medium is dry before the antimicrobial disks are applied.** Plates may be dried in an incubator or biohazard hood as described in step *e*. (If it takes longer than 15 minutes for the inoculum to dry, use a smaller volume / express more suspension from the swab in the future.)

h) Using sterile forceps, tweezers or a disk dispenser, apply disks of the selected antimicrobial agents to the surface of the inoculated medium; tap them to ensure they are in complete contact with the surface of the agar. **Once a disk has touched the agar surface, diffusion begins and it must not be moved.** All disks should be placed approximately the same distance from the edge of the plate and from each other (Figure 28).

i) Cover and invert the inoculated plates and incubate them at 35˚–36.5˚C in a 3%–5% CO_2 atmosphere (in a CO_2-incubator or candle-extinction jar) for 20–24 hours.

j) At 20–24 hours after inoculation and incubation, read the results of the antimicrobial susceptibility tests.
   - Examine the plates from the back, viewed from the top down against a black background and illuminated with reflected light (so hazy growth is more easily seen). Measure the diameter of the zone of inhibition with calipers, a ruler, or a ruler on a stick (see Figure 6).
   - Read the results for ATCC 49226 and compare them to the values in Table 9; if they are in control, continue reading and comparing results for the other

QC strains tested. If these are also in control, continue to read and record results for the clinical isolates.

k) Interpret the results. Table 10 presents zone inhibition diameters and equivalent minimal inhibitory concentrations (MICs) for test strains, along with the NCCLS standard interpretations of those zones diameters as sensitive, intermediate, or resistant.

After interpreting results, report them back to the primary laboratory.

Antimicrobial susceptibility testing of N. gonorrhoeae *by Etest® antimicrobial gradient strip*

Antimicrobial susceptibility testing with the Etest® antimicrobial gradient strip[29] is technically as simple to perform as the disk diffusion test, but provides semi-quantitative MIC results. The strip is impregnated with a standard gradient of antimicrobial agent, and the front of the strip has MIC values that are to be read in correspondence with inhibition of growth on the plate after incubation. **Always read the insert** in the package of Etest® strips, and **follow the manufacturer's instructions** for performance of the test.

Antimicrobial susceptibility testing of *N. gonorrhoeae* is performed on GC base medium plus 1% defined growth supplement; methods for the preparation and QC of this medium are included in Appendix 2 ("Media, Reagents and Quality Control"). The standardization of the inoculum and methods for the inoculation of the test plate are the same for the Etest® as they are for the disk diffusion test for *N. gonorrhoeae*; follow steps *a* through *g* above, and then continue with step *h*, below. Strict quality control practices are of extreme importance in order for the proper performance and appropriate interpretation of the antimicrobial susceptibility test. If conditions cannot be controlled and standardized, it is better that the laboratory not perform the antimicrobial susceptibility test at all, because the results obtained cannot be interpreted according standardized criteria. Inaccurate results are useless to the clinician, can even cause harm to a case-patient, and should not be recorded for use in public health policy treatment decision-making.

Laboratorians should ensure that the Etest® strips used for antimicrobial susceptibility testing of *N. gonorrhoeae* strains cover the appropriate range of antibiotic concentrations for these organisms.[30]

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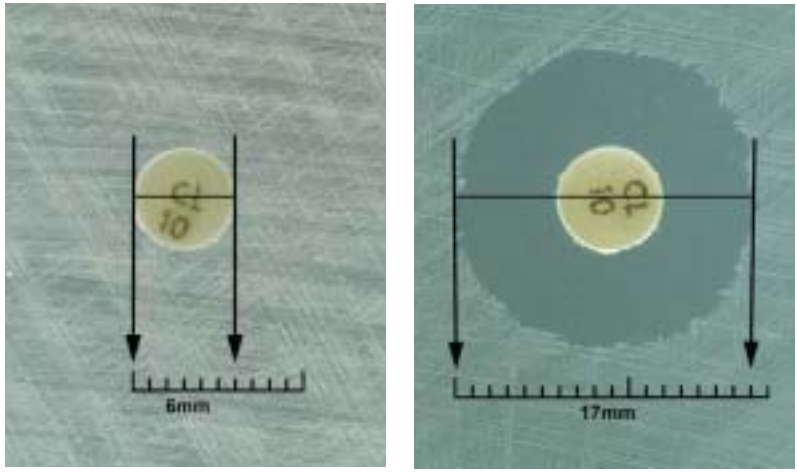
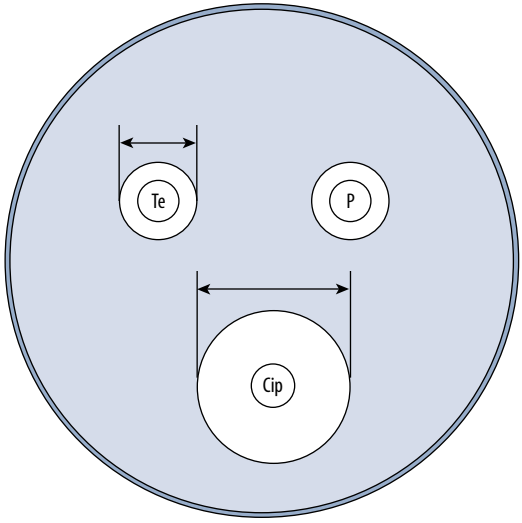
[29] The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor settings (see Appendix 13).

[30] Please note that for certain antimicrobial agents (particularly some ß-lactams), the Etest® is available in a high- and low range of concentrations. For testing of *N. gonorrhoeae* with the ceftriaxone Etest®, for example, it is recommended that laboratories use the low-range concentration (0.002 µg/ml – 32 µg/ml) rather than the high-range concentration (0.016 µg/ml – 256 µg/ml). A complete list of strips and ranges of concentrations is available from AB Biodisk (at http://www.abbiodisk.se/productsservice/product.htm).

FIGURE 28: Disk diffusion testing: disk placement for ***Neisseria gonorrhoeae*** and measurements of inhibition zone diameters



Top: Photographs of bacterial growth, zones of inhibition, and measurement of the zones. Note that the disk on the left is surrounded by a resistant strain and the diameter of the zone of inhibition is equivalent to the diameter of the disk (6 mm), whereas the figure on the right shows a strain with a zone of inhibition of 17 mm.



Bottom: The shaded area represents uniform growth of the strain on the plate; the white areas surrounding the disks represent zones of inhibition. Zones of inhibition are measured as indicated by the double-arrow lines.

Note: Calipers or a ruler on a stick (*see Figure 6*) can be helpful for measuring the diameter of a zone of inhibition.

TABLE 9: Acceptable limits for MICs and inhibition zone diameters for quality control strains of ***Neisseria gonorrhoeae***

Antimicrobial agent (disk concentration)	Diameter of disk diffusion zone of inhibition (mm) and equivalent minimal inhibitory concentration (MIC) [µg/ml] [a]							
	Strain [b]	ATCC 49226 [c]	F-28	P681E	CDC 10,328	CDC 10,329	SPJ-15	SPL-4
	Phenotype	***Susceptible***	***SpcR***	***PP-TR***	***CipI***	***CipR***	***"AznR"***	***"CfxDS"***
Penicillin (10-unit)	Disk (mm) *[MIC µg/ml]*	26 – 34 *[0.25 – 1.0]*	37 – 47 *[0.015 – 0.06]*	6 – 10 *[4.0 – 64.0]*	<NT> *[32.0 – ≥ 64.0]* [e]	<NT> *[32.0 –≥ 64.0]* [e]	<NT> *[0.5 – 1.0]*	<NT> *[4.0 – 8.0]*
Tetracycline (30-µg)	Disk (mm) *[MIC µg/ml]*	30 – 42 *[0.25 – 1.0]*	35 – 40 *[0.125 – 0.5]*	14 – 19 *[8.0 – 32.0]*	<NT> *[0.5 – 1.0]*	<NT> *[2.0 – 8.0]*	<NT> *[1.0 – 4.0]*	<NT> *[2.0 – 8.0]*
Spectinomycin (100-µg)	Disk (mm) *[MIC µg/ml]*	23 – 29 *[8.0 – 32.0]*	6 – 7 *[≥ 128.0]*	22 – 25 *[< 128.0]*	<NT> *[< 128.0]*	<NT> *[< 128.0]*	<NT> *[< 128.0]*	<NT> *[< 128.0]*
Ceftriaxone (30-µg)	Disk (mm) *[MIC µg/ml]*	39 – 51 *[0.004 – 0.016]*	49 – 62 *[0.0005 – 0.004]*	43 – 53 *[0.002 – 0.008]*	<NT> *[≤0.002 – 0.008]* [d]	<NT> *[0.004 – 0.015]*	<NT> *[0.004 – 0.015]*	<NT> *[0.06 – 0.125]*
Cefixime (5-µg)	Disk (mm) *[MIC µg/ml]*	37 – 45 *[0.004 – 0.03]*	<NT> *[0.001 – 0.008]*	<NT> *[0.004 – 0.03]*	<NT> *[0.008 – 0.06]*	<NT> *[0.008–0.125]*	<NT> *[0.008 – 0.06]*	<NT> *[0.25 – 0.5]*
Ciprofloxacin (5-µg)	Disk (mm) *[MIC µg/ml]*	48 – 58 *[0.001 – 0.008]*	40 – 55 *[≤0.002 – 0.008]* [d]	45 – 55 *[≤0.001 – 0.004]* [d]	30 – 34 *[0.25 – 0.5]*	21 – 26 *[1.0 – 2.0]*	<NT> *[0.002 – 0.015]*	<NT> *[8.0 – 32.0]*
Ofloxacin (5-µg)	Disk (mm) *[MIC µg/ml]*	43 – 51 *[0.004 – 0.016]*	40 – 55 *[0.004 – 0.015]*	40 – 50 *[0.004 – 0.015]*	27 – 32 *[0.25 – 1.0]*	18 – 21 *[2.0 – 4.0]*	<NT> *[0.008 – 0.03]*	<NT> <*[ND]*>
Azithromycin (15-µg)	Disk (mm) *[MIC µg/ml]*	<NT> *[0.125 – 0.5]* [c]	<NT> *[0.03 – 0.125]*	<NT> *[0.03 – 0.06]*	<NT> *[0.03 – 0.06]*	<NT> *[0.125 – 0.5]*	19 – 22 *[1.0 – 4.0]*	<NT> *[0.125 – 0.5]*
ß-lactamase production	(+ / –)	–	–	+	+	+	–	–

[a] These results were developed on a GC susceptibility test medium of GC II agar base medium plus 1% IsoVitaleX; the ranges presented here may vary with different formulations of GC agar base and growth supplement.

[b] These QC strains can be obtained from the Gonorrhea Research Branch, CDC. (See Appendix 14 for an address.)

[c] ATCC 49226 is the NCCLS-recommended quality control strain. MICs and inhibition zone diameters for ATCC 49226 are those recommended by the NCCLS, except for MICs for azithromycin which were derived from interlaboratory testing by six laboratories.

[d] Reference laboratories have not tested these QC strains against a concentration of ciprofloxacin lower than the lowest MIC shown.

[e] Reference laboratories have not tested these QC strains against a concentration of penicillin higher than the highest MIC shown.

<NT> indicates not tested by disk diffusion methods; <[ND]> indicates not tested by MIC methods.

TABLE 10: Interpretive criteria for antimicrobial susceptibility of ***Neisseria gonorrhoeae***

Antimicrobial agent	Disk potency	Breakpoints for zone of inhibition (mm) and equivalent MIC (µg/ml) [a]			NCCLS QC strain ***N. gonorrhoeae*** ATCC 49226
		Susceptible	Intermediate	Resistant	
Penicillin	10 units	≥ 47 mm *(≤ 0.06 µg/ml)*	27 – 46 mm *(0.125 – 1.0 µg/ml)*	≤ 26 mm *(≥ 2.0 µg/ml)*	26 – 34 mm *(0.25 – 1.0 µg/ml)*
Tetracycline	30 µg	≥ 38 mm *(≤0.25 µg/ml)*	31 – 37 mm *(0.5 – 1.0 µg/ml)*	≤ 30 mm *(≥ 2.0 µg/ml)*	30 – 42 mm *(0.25 – 1.0 µg/ml)*
Spectinomycin	100 µg	≥ 18 mm *(≤ 32.0 µg/ml)*	15 – 17 mm *(64.0 µg/ml)*	≤ 14 mm *(≥ 128.0 µg/ml)*	23 – 29 mm *(8.0 – 32.0 µg/ml)*
Ceftriaxone **	30 µg	≥ 35 mm *(≤ 0.25µg/ml)*	** **	** **	39 – 51 mm *(0.004 – 0.016 µg/ml)*
Cefixime **	5 µg	≥ 31 mm *(≤ 0.25 µg/ml)*	** **	** **	37 – 45 mm *(0.004 – 0.03 µg/ml)*
Ciprofloxacin	5 µg	≥ 41 mm [b] *(≤ 0.06 µg/ml)*	28 – 40 mm *(0.125 – 0.5 µg/ml)*	≤ 27 mm *(≥ 1.0 µg/ml)*	48 – 58 mm *(0.001 – 0.008 µg/ml)*
Ofloxacin	5 µg	≥ 31 mm *(≤ 0.25 µg/ml)*	25 – 30 mm *(0.5 – 1.0 µg/ml)*	≤ 24 mm *(≥ 2.0 µg/ml)*	43 – 51 mm *(0.004 – 0.016 µg/ml)*

** Only "susceptible" interpretive criteria are available for zones and MICs for ceftriaxone and cefixime; isolates with ranges outside the values in this table should be noted as having "decreased susceptibility" and sent to an international reference laboratory for further testing.

[a] *Source:* NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA.

[b] Recent experience has shown that some gonococcal isolates with ciprofloxacin zone sizes of 36 mm (and therefore classified as "intermediate" by current NCCLS criteria) have MICs of 0.06 mg/ml and are classified as "susceptible" by current NCCLS criteria for MICs determined by agar dilution susceptibility testing. More research is needed to clarify the relationship between an MIC of 0.06 µg/ml of ciprofloxacin and the corresponding disk diffusion zone inhibition diameters exhibited by such organisms. It is therefore advised that the antimicrobial susceptibilities of isolates exhibiting inhibition zone diameters of 36–41 mm be confirmed by MIC testing before they are classified as exhibiting intermediate resistance to ciprofloxacin.

Methods

a - g) Methods for the preparation of the standard inoculum and the inoculation of the test plates are included in steps *a* through *g* of 'disk diffusion methods', listed above.

h) Remove the Etest® strips from the freezer, and allow them to reach room temperature (approximately 30 minutes). It is extremely important to **keep the Etest® strips that are not going to be used in a freezer at -20˚C**.

i) When the surface of the plate is dry, place the Etest® strips on the agar surface with sterile forceps, tweezers or test-dispenser, as illustrated in Figure 7. (Make sure that the printed MIC values are facing upward, *i.e.*, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar.) Once the test strip has touched the surface, it should not be moved.

- Although the manufacturer's insert for the Etest® says that up to two strips can be used on a 100-mm plate and up to six on a 150-mm plate, because gonococci can have such wide zones of inhibition, **this laboratory manual advises using only one Etest® strip per 100 mm plate for *N. gonorrhoeae*.** The number of strips on a 150-mm plate will be determined by the combination of drugs being tested; zones of inhibition *must not* overlap. (Once laboratorians have determined the range of susceptibilities of local gonococcal isolates to various antimicrobial agents with the Etest® on 100-mm plates, they can assess which combinations of antimicrobial agents can be tested on a 150-mm plate without overlapping zones of inhibition, usually 3 or 4 antimicrobial agents.)

j) Incubate the inoculated Etest® plate according to the manufacturer's instructions (usually 20–24 hours at 35°–36.5°C in a 5% CO_2 atmosphere).

k) After incubation for 20–24 hours, there will be an ellipse of inhibition of bacterial growth on the plate around the Etest® strip, and the MIC values can be read (see Figure 8).
   - The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of a gonococcal isolate to ciprofloxacin, an MIC recorded from the graduations on the Etest® strip might be 0.094 µg/ml; however, the reported MIC would be 0.125 µg/ml and the organism would be interpreted as exhibiting intermediate resistance to ciprofloxacin.

l) Read the results for ATCC 49226 and compare them to the values in Table 9; if they are in control, continue reading and comparing results for the other QC strains tested. If these are also in control, continue to read and record results for the clinical isolates. **It is essential to review the MIC results of the quality control strains prior to interpreting the MICs of the clinical isolates.**

m) Read and interpret the results for the test strains. Table 10 presents the NCCLS interpretive criteria (susceptible, intermediate, resistant) for different antimicrobials, including those currently recommended for the primary therapy of uncomplicated gonorrhea.

A reading guide for interpretation of Etest® antimicrobial susceptibility results and guidance in reading MICs from the Etest® strip is presented in Figure 8. The guide, included with the permission of AB Biodisk, shows how growth appears around the strip and provides guidance for how the test should be interpreted.

TABLE 11: MIC critical values for ***Neisseria gonorrhoeae*** and appropriate laboratory response

Antimicrobial agent	Therapeutic dose	Critical MIC value (disk diffusion zone size)	Phenotypic 'resistance' category	Response [a]
Ceftriaxone	125 mg, single dose, intramuscular (IM)	MIC > 0.25 µg/ml *(<35 mm)*	Decreased susceptibility *(CroDS)*	Isolates showing higher MICs or smaller zone sizes thanthese ranges should be retested to confirm the results, preferably with QC strains specifically for the antimicrobial agent showing abnormally high values. A confirmed MIC value greater than or equal to the critical MIC should alert laboratorians and clinicians to ascertain that infections caused by such strains (and treated with the WHO-recommended dose of the corresponding agent) responded successfully to therapy. If, after re-testing, the high MIC is confirmed, an international reference laboratory should be notified and arrangements made to send the isolate for further confirmation.
Cefixime	400 mg, single dose, oral	MIC > 0.25 µg/ml *(<31 mm)*	Decreased susceptibility *(CfxDS)*	
Ciprofloxacin	500 mg, single dose, oral	MIC 0.125 – 0.5 µg/ml *(28 - 40 mm)*	Intermediate resistance *(CipI)*	
		MIC ≥ 1.0 µg/ml *(≤ 27mm)*	Resistance *(CipR)*	
Ofloxacin	400 mg, single dose, oral	MIC 0.5 – 1.0 µg/ml *(25 – 30 mm)*	Intermediate resistance *(OfxI)*	
		MIC ≥ 2.0 µg/ml *(≤ 24 mm)*	Resistance *(OfxR)*	
Azithromycin	2 g, single dose, oral	MIC ≥ 1.0 µg/ml [b] *(≤ 25 mm)* [b]	'Resistance' [b] *('AznR')*	
Spectinomycin	2 g, single dose, intramuscular (IM)	MIC ≥ 128.0 µg/ml *(≤ 14 mm)*	Resistance *(SpcR)*	

[a] If MICs greater than or equal to the critical MIC have already been confirmed in a region, it may be desirable to confirm the MIC with regard to evaluating treatment outcome for patient management purposes; however, it is not necessary to re-contact an international reference laboratory.

[b] NCCLS [2002] has not recommended criteria for the interpretation of susceptibilities of *N. gonorrhoeae* to azithromycin. Because there are limited outcome data corresponding to treatment of gonococcal infections with azithromycin (2 g), it is suggested that laboratorians and clinicians collaborate to correlate treatment outcome data with *in vitro* susceptibility results to establish an interpretive breakpoint for resistance corresponding to a 5% treatment failure rate. In the absence of conclusive clinical outcome data, this document presents a 'working' interpretive criterion for resistance based on a limited number of treatment failures and laboratory *in vitro* agar dilution and disk diffusion antimicrobial susceptibility data [Handsfield *et al.* 1994; CDC, unpublished data]: *N. gonorrhoeae* strains with a confirmed MIC of ≥1.0 µg/ml exhibit an *in vitro* 'resistant' phenotype to azithromycin.

- ***Note:*** Although the WHO-recommended dose for treatment of uncomplicated gonorrhea with azithromycin is 2 grams in a single oral dose, because a 1-gram single oral dose of azithromycin is recommended for the treatment of genital *Chlamydia trachomatis* infections, this dose may be used incidentally to treat gonococcal co-infections. Evaluation of clinical treatment outcomes has indicated that gonococcal infections caused by strains with MICs of ≥0.125 µg/ml may fail to respond to treatment with the 1-gram dose of azithromycin [Young *et al.* 1997].

When reading any antimicrobial susceptibility test results for *N. gonorrhoeae*, the laboratorian should be aware of critical values that indicate a need for retesting. Table 11 presents a listing of critical antimicrobial susceptibility test values for the laboratory to be aware of. If the MIC results for an organism are higher than those listed for the specific antimicrobial agent, the reference laboratory should re-test the isolate. If the results are still atypical, confirm the identification of the organism, ensure the test is being performed properly, and then re-test the isolate again. If it still produces a high MIC, notify the national and an international reference laboratory and send the isolate for further investigation. Instructions for the preservation and storage of isolates are presented in Appendix 11, while Appendix 12 includes the instructions for regulation-compliant packaging and shipping of isolates.

When the susceptibility value is confirmed upon re-test, the confirmatory laboratory should notify the submitting laboratory and then other laboratories in the regional and international network. If the isolate represents a new antimicrobial resistance phenotype, it is important that the confirming reference laboratory disseminate preserved cultures of the isolate to other reference laboratories for inclusion among susceptibility quality control strains. Isolates showing a previously undescribed resistance pattern should not be used for scientific research (such as the determination of the resistance mechanisms) without permission from the originating clinician and/or laboratory.

Data for decision-making

Antimicrobial susceptibility testing can be performed on an isolate presumptively identified as *N. gonorrhoeae*, although confirmatory testing should be completed before antimicrobial susceptibility test results exceeding the critical MIC values are reported. (For example, before reporting results, laboratories should confirm the identification of an organism showing an unexpectedly high MIC to, *e.g.*, ceftriaxone.) Once the laboratory has determined the antimicrobial susceptibilities, the information should be reported back to public health officials in a timely manner. Factors to consider in the development of a treatment policy include:

- Laboratories should screen and report values for antimicrobial agents currently in use for primary therapy of gonorrhea in the region and, ideally, also for the second line drugs.
- MIC "critical values" can be useful tools to initiate (enhanced) surveillance and epidemiological investigations to determine if there is an association between the *in vitro* susceptibility of a strain and the clinical outcome.
- Extended-spectrum cephalosporins, fluoroquinolones, and spectinomycin are recognized as the most effective antimicrobial agents for the treatment of gonorrhea in most geographic areas of the world.

- The antimicrobial agent and dose chosen should be effective against at least 95% of local gonococcal strains.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be readily available.
- It should be possible to store the chosen antimicrobial agent under conditions (*e.g.*, refrigeration) that will maintain the activity of the drug.

It is important to consider the above factors when making decisions relating to treatment of gonorrhea. Determination of antimicrobial susceptibilities to therapeutic agents will help public health officials review the appropriateness of treatment recommendations for local populations, and surveillance of antimicrobial susceptibilities will promote effective disease control.

Bacterial Agents of Enteric Diseases of Public Health Concern

Salmonella serotype Typhi
Shigella
Vibrio cholerae

CHAPTER VII

Salmonella serotype Typhi

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Salmonella serotype Typhi (*S.* Typhi), the etiologic agent of typhoid fever, causes an estimated 16.6 million cases and 600,000 deaths worldwide each year. A syndrome similar to typhoid fever is caused by "paratyphoidal" serotypes of *Salmonella*. The paratyphoid serotypes (*i.e.*, *S.* Paratyphi A, *S.* Paratyphi B, and *S.* Paratyphi C) are isolated much less frequently than *S.* Typhi. Rarely, other serotypes of *Salmonella*, such as *S.* Enteritidis, can also cause "enteric fever." Like other enteric pathogens, *S.* Typhi is transmitted through food or water that has been contaminated with feces from either acutely infected persons, persistent excretors, or from chronic asymptomatic carriers. Humans are the only host for *S.* Typhi; there are no environmental reservoirs.

Effective antimicrobial therapy reduces morbidity and mortality from typhoid fever. Without therapy, the illness may last for 3–4 weeks and case-fatality rates may exceed 10%. With appropriate treatment, clinical symptoms subside within a few days, fever recedes within 5 days, and mortality is reduced to approximately 1%. Relapses, characterized by a less severe but otherwise typical illness, occur in 10%–20% of patients with typhoid fever, usually after an afebrile period of 1–2 weeks. Relapses may still occur despite antimicrobial therapy.

S. Typhi is most frequently isolated from blood during the first week of illness, but it can also be present during the second and third weeks of illness, during the first week of antimicrobial therapy, and during clinical relapse. Fecal cultures are positive in approximately half the cases during the first week of fever, but the largest number of positive cultures occurs during the second and third weeks of disease. Bone marrow cultures are frequently positive (90% of cases) and are more likely to yield *S.* Typhi than are cultures from any other site, especially when the patient has already received antimicrobial therapy. Organisms can also be isolated from duodenal aspirates, rose spots, and infrequently (*i.e.*, in approximately 25% of cases) from urine cultures.

In typhoid fever, serologic responses to O, H, and Vi antigens usually occur by the end of the first week of illness. The Widal test, which measures antibody responses to H and O antigens, can suggest the diagnosis, but the results are not definitive and must be interpreted with care because titers also may be elevated in response to a number of other infections. High-titer, single serum specimens from adults living in areas of endemic disease have little diagnostic value. Even when paired sera are used, the results must be interpreted in light of the patient's history of typhoid immunization and previous illness, the stage of the illness when the first serum specimen was obtained, the use of early antimicrobial therapy, and the reagents used.

There are currently (2002) at least two effective vaccines available for typhoid, both of which were recently licensed for use in the United States. The oral live attenuated vaccine (for use in children aged 6 years and older) and the parenteral (*i.e.*, injectable) capsular polysaccharide vaccine (for use in children aged 2 years and older) each have efficacy of 50%–80% and fewer adverse events associated with their use than earlier typhoid vaccines. A team doing research in Vietnam reported promising preliminary success of a new conjugate vaccine in early 2001. The two U.S.-licensed vaccines have been widely and effectively used by travelers to typhoid-endemic regions, though the expense and limited experience with their use as a public health intervention in countries with high endemic rates of typhoid fever precludes the widespread use of these vaccines in countries with limited resources. Nonetheless, it is good policy for laboratory technicians who may be working with this organism to supplement their laboratory safety practices and ensure that their vaccination status against typhoid fever remains current.

In developing countries, typhoid fever is frequently diagnosed solely on clinical grounds; however, isolation of the causative organism is necessary for a definitive diagnosis. Isolation of the agent is also a necessity for the performance of antimicrobial susceptibility testing.

Resistance to the antimicrobial agents amoxicillin, trimethoprim-sulfamethoxazole, and chloramphenicol is being increasingly reported among *S.* Typhi isolates; quinolone resistance has been reported from the Indian subcontinent and Southeast Asia. **Determining antimicrobial resistance patterns is essential in recommending treatment.** In areas where resistance to these agents is common among circulating *S.* Typhi strains, fluoroquinolones and parenteral third-generation cephalosporins are probably the best choice for empiric treatment of typhoid fever. Cefixime may be recommended in some cases as a less expensive, oral alternative to parenteral ceftriaxone.

Identification of *S.* Typhi

A preliminary report of typhoid can be issued to a clinician as soon as a presumptive identification of *S.* Typhi is obtained. Methods for the isolation of

S. Typhi from normally sterile sites (*e.g.*, blood, bone marrow, and urine) are presented in Appendix 3; isolation of *S.* Typhi from fecal specimens is presented in Appendix 10. Blood, bone marrow, or urine specimens collected from a patient with suspect typhoid fever or a diagnosis of fever of unknown origin and sent to a laboratory should be cultured on blood or chocolate agar; in addition, if resources permit the use of more than one medium, MacConkey agar (MAC) should be inoculated. Fecal specimens should be cultured on selective agar media (*e.g.*, bismuth sulfite agar [BS] or desoxycholate citrate agar [DCA]). Isolates from blood, bone marrow or urine should be Gram stained, whereas isolates obtained from stool specimens should not. In most situations, presumptive identification is based on the reaction of the isolate on Kligler iron agar (KIA) / triple sugar iron Agar (TSI) **and** a positive serologic reaction in *Salmonella* Vi or D antisera.

If gram-negative rods are cultured from specimens obtained from normally sterile sites and/or their culture yields colorless colonies on MAC, the laboratorian should inoculate KIA/TSI. Isolates that have a reaction typical of *S.* Typhi on KIA/TSI should then be tested with Vi and D antisera. The results of the serologic testing should be promptly reported to health authorities, and Mueller-Hinton agar should be inoculated for antimicrobial susceptibility testing. For **any** blood isolate, antimicrobial susceptibility testing should not be delayed pending biochemical or serologic identification.

Although clinicians will not necessarily be waiting for the results of antimicrobial susceptibility tests or even the verification of identification, the reference laboratory should confirm the pathogen's identification via biochemical and serologic characterization and record these and the antimicrobial susceptibility results along with the patient's demographic information for epidemiologic purposes. A flowchart of tests for the identification of an agent as *S.* Typhi is presented in Figure 29, and Figure 30 illustrates a worksheet to record laboratory data.

Kligler iron agar and triple sugar iron agar

Suspicious colonies should be carefully picked from plating media to a screening medium such as Kligler iron agar (KIA) or triple sugar iron agar (TSI) or to any nonselective agar medium and then incubated overnight. Select at least one of each colony type of the well-isolated colonies on each plate. Using an inoculating needle, lightly touch **only the center of the colony.** Do not take the whole colony or go through the colony and touch the surface of the plate because this practice could result in picking up contaminants that may be present on the surface of the agar. If the ability to select an isolated, pure colony is questionable, the suspicious colony should be purified by streaking for isolation on another agar plate before inoculating the colony to a TSI/KIA slant.

TSI and KIA are inoculated by stabbing the butt and streaking the surface of the slant. The caps should be loosened before incubation. After incubation for 24

FIGURE 29: Flowchart for the isolation and identification of *Salmonella* ser. Typhi

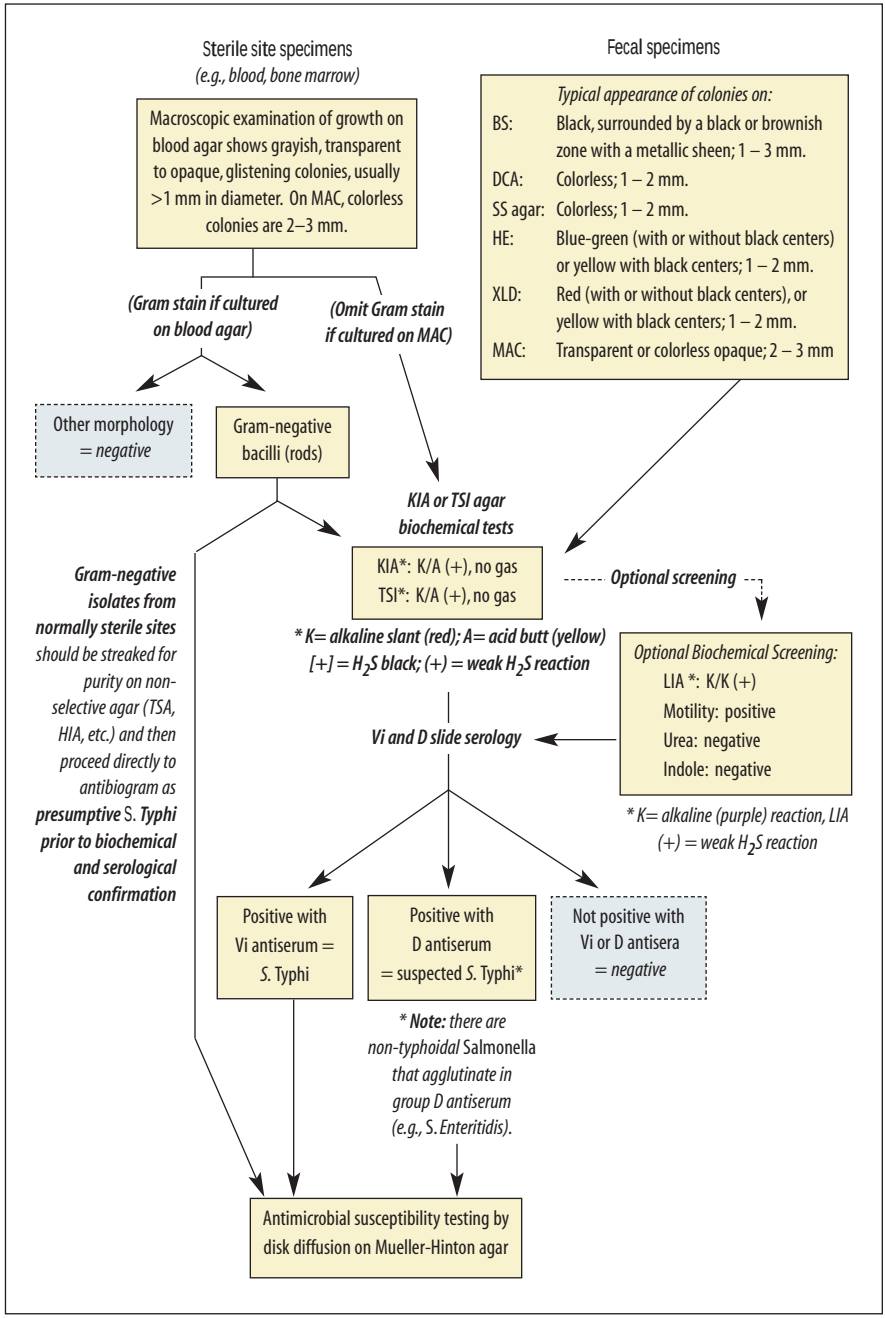


FIGURE 30: Sample worksheet for ***Salmonella*** ser. Typhi test results

Specimen number	Agar medium [a]	Colony [b]	Gram stain [c]	KIA / TSI	Optional					Slide Serology [d]		Identification
					LIA	Motility	Urea			Vi	D	
		1										
		2										
		3										
		1										
		2										
		3										
		1										
		2										
		3										
		1										
		2										
		3										
		1										
		2										
		3										

[a] The choice of agar medium will depend on whether a specimen is from a normally sterile site (*e.g.,* blood, bone marrow, urine) or is a fecal specimen. Sterile site specimens should be cultured on blood agar; fecal specimens should be plated on selective media (such as BS, DCA, SS, HE, XLD, or MAC).

[b] If macroscopic examination of morphology reveals there is more than one type of colony on a plate, perform tests to identify each of the different isolates.

[c] Gram stain should only be performed on isolates from normally sterile sites (*e.g.,* blood, bone marrow, urine) and should not be performed on growth from MAC or other selective media.

[d] If prompt identification is required for clinical decision-making, slide serology may precede biochemical testing.

hours at 35˚–37˚C, the TSI or KIA slants are observed for reactions typical of *Salmonella.* **On TSI or KIA slants, *S.* Typhi characteristically produces an alkaline slant (red, "K"), an acid butt (yellow, "A"), and a small amount of blackening of the agar (H_2S, +) at the site of the stab on the slant and in the stab line (Figure 31); no gas (G) is produced.** It is worth noting that occasionally *S.* Typhi isolates do not produce H_2S. *S.* Paratyphi A isolates on TSI/KIA are usually K/AG and do not produce H_2S. Most other *Salmonella* serotypes produce a K/AG+ reaction, indicating that glucose is fermented with gas and H_2S production. Table 12 summarizes the reactions of *Salmonella* on screening biochemicals.

Additional screening biochemicals for the identification of *S.* Typhi

Isolates can be identified biochemically as *Salmonella* by traditional tubed media or commercial biochemical systems. Table 12 lists biochemical reactions of the tests that are helpful screening tests for *S.* Typhi. After performing the tests, read and record the results, then compare them to results for presumptive *S.* Typhi. If they match, then proceed by confirming with serologic testing if it has not already been performed.

Lysine iron agar

Lysine iron agar (LIA) is a useful screening medium because most *Salmonella* isolates decarboxylate lysine and produce H_2S, whereas gas production varies by serotype. Preparation and quality control (QC) of this medium are described in Appendix 2. Inoculate LIA by stabbing the butt and streaking the surface of the slant; read and interpret the reactions after incubation for 24 hours at 35˚–37˚C for 24 hours.

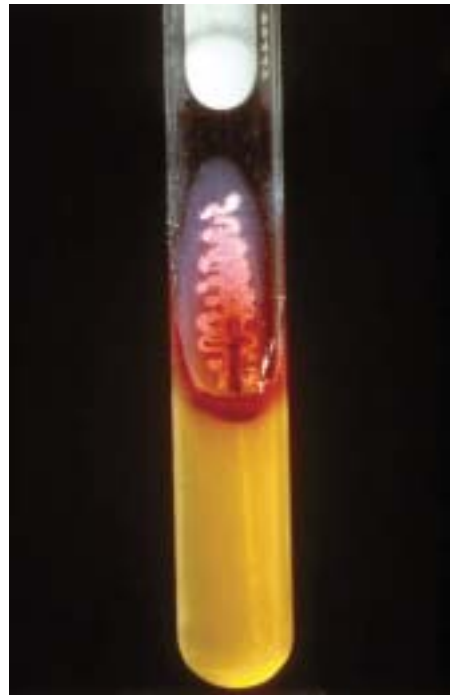
On LIA, *Salmonella* typically give an alkaline (purple) reaction on the slant and butt and may produce gas and H_2S (blackening of medium) as well, as indicated in Table 12. When the reaction in the butt of the tube is alkaline, the lysine is decarboxylated and the isolate is termed "lysine-positive." Unlike most other *Salmonella*, *S.* Paratyphi A isolates are lysine-negative and appear yellow on LIA.

If a diagnosis of infection with *S.* Typhi is suspected and prompt diagnosis is required to identify appropriate treatment, suspect isolates should be screened with antisera prior to biochemical identification. However, in the setting of a public health study, because slide serology can be performed using growth from KIA, TSI, or LIA, performing serology after those tests and saving antiserum for only those isolates showing biochemical characteristics typical of *S.* Typhi is more cost-effective.

Motility agar

Motility agar should be inoculated with a straight inoculating needle, making a single stab about 1–2 cm down into the medium. The surface of the motility agar

FIGURE 31: ***Salmonella*** ser. Typhi colonies on triple sugar iron (TSI) Agar



On triple sugar iron agar (TSI) or Kligler iron agar (KIA) slants, *S.* Typhi characteristically produces an alkaline slant (red, "K"), an acid butt (yellow, "A"), and a small amount of blackening of the agar (H_2S, +) at the site of the stab on the slant and in the stab line; no gas (G) is produced.

should be dry when used: moisture can cause a non-motile organism to grow down the sides of the agar creating a haze of growth and appearing to be motile. Motility agar may be inoculated with growth from a KIA or TSI that shows a reaction typical of *S.* Typhi. Alternatively, motility agar can be inoculated at the same time as the KIA or TSI slant by using the same inoculating needle without touching the colony again. (When motility agar is to be inoculated at the same time as KIA or TSI, use the same colony to first inoculate the motility agar and then to inoculate the KIA or TSI by stabbing the butt and then streaking the surface of the slant. **Do not select a second colony to inoculate the KIA or TSI after the motility agar has been inoculated because it may represent a different organism.**

TABLE 12. Typical reactions of ***Salmonella*** spp. in screening biochemicals

Screening medium	***Salmonella*** Typhi	***Salmonella*** Paratyphi A	Nontyphoidal ***Salmonella*** or ***Salmonella*** Paratyphi B or C
Triple sugar iron agar (TSI)	K/A(+)[a]	K/AG-[a]	K/AG+[a]
Kligler iron agar (KIA)	K/A(+)[a]	K/AG-[a]	K/AG+[a]
Lysine iron agar (LIA)	K/K(+)[b]	K/AG-[b]	K/K+[b]
Hydrogen sulfide (H_2S)	(weak)[c]	negative	positive
Urea	negative	negative	negative
Motility	positive[c]	positive	positive
Indole	negative	negative	negative

[a] for KIA / TSI: K = alkaline (red); A = acid (yellow); G = gas production; + = black H_2S produced (weak); – = no H_2S

[b] for LIA: K= alkaline (purple); A= acid (yellow); G = gas production; + = black H_2S produced (weak); – = no H_2S
~ An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated.
~ An acid reaction (yellow) in the butt of the medium indicates that lysine was not decarboxylated.

[c] this reaction occurs 97% of the time.

Examine after overnight incubation at 35°–37°C. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 39). Non-motile organisms do not grow out from the line of inoculation. Motility reactions may be difficult for inexperienced laboratorians to read; therefore, reactions should be compared with positive and negative control strains. *S.* Typhi is usually motile (+ 97%).

Sulfide-indole-motility medium is a combination medium that is commercially available in dehydrated form (see Appendix 2, "Media, Reagents, and Quality Control"). It can be used in place of motility medium.

Urea medium

Urea medium screens out urease-producing organisms (*e.g.*, *Klebsiella* and *Proteus*). Urea agar is inoculated heavily over the entire surface of the slant. Loosen caps before incubating overnight at 35°–37°C. **Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color** (Figure 40). Urease-negative organisms do not change the color of the medium, which is a pale yellowish-pink. *S.* Typhi is always urease negative.

Slide serology for *S.* Typhi identification

TSI/KIA cultures that are suspicious for *S.* Typhi should be screened serologically with *Salmonella* Vi and group D "O" antisera. Because Vi is a capsular antigen, if it is present, it may mask the somatic "O" group reaction. Therefore, *S.* Typhi isolates will usually either be positive in the Vi or the D antisera (though it is possible they

will be weakly positive in both). Occasionally, *S.* Paratyphi C will also be positive in Vi antiserum, but because it produces gas from glucose and is H_2S positive, reactions on KIA/TSI allow for the differentiation of *S.* Paratyphi C from *S.* Typhi.

Serologic agglutination tests may be performed in a petri dish or on a clean glass slide.

a) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, LIA, or other nonselective agar medium. Serologic testing should not be done on growth from selective media (*e.g.*, MAC, DCA, BS, or XLD) because selective media may yield false-negative serologic results.

b) Emulsify the growth in three small drops of physiological saline and mix thoroughly.

c) Add a small drop of O group D antiserum to one of the suspensions and a small drop of Vi antiserum to a second. The third suspension is used as a control for autoagglutination (roughness). Usually approximately equal volumes of antiserum and growth suspension are mixed, but the volume of suspension may be as much as double the volume of the antiserum. To conserve antiserum, volumes as small as 10 ml can be used. A bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32).

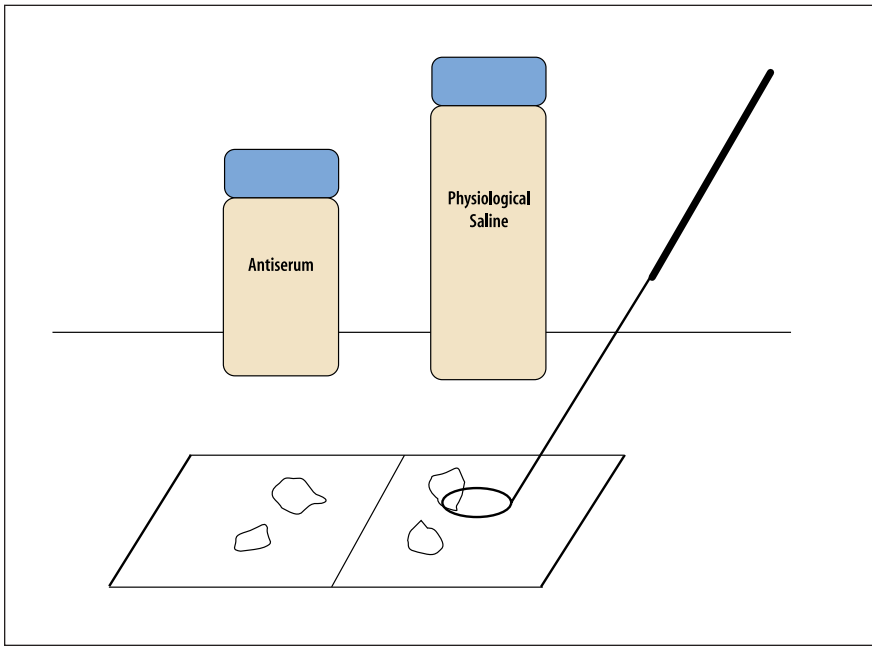
d) Mix the suspension and antiserum thoroughly and then tilt the slide back and forth to observe for agglutination. It will be easier to see the agglutination if the slide is observed under a bright light and over a black background; if the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 42). Examine the saline suspension carefully to ensure that it is even and does not show clumping caused by autoagglutination. If autoagglutination occurs, the culture is termed "rough" and cannot be serotyped. Strong agglutination reactions are read as positive.

Cultures that have a TSI/KIA reaction typical of *S.* Typhi and that react serologically in either the Vi or the D antisera can be presumptively identified as *S.* Typhi. The tube agglutination for the "d" flagellar antigen or further biochemical tests may be conducted by reference laboratories to confirm the identification as *S.* Typhi.

Antimicrobial susceptibility testing of *S.* Typhi

Treatment with an appropriate antimicrobial agent is crucial for patients with typhoid. Because recent reports have noted an increasing level of resistance to one or more antimicrobial agents in *S.* Typhi strains, isolates should undergo antimicrobial susceptibility testing as soon as possible. The disk diffusion method

FIGURE 32: Use of a bent loop to dispense small amounts of antiserum for slide agglutination tests



presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),[31] and, if performed precisely according to the protocol below, will provide data that can reliably predict the *in vivo* effectiveness of the drug in question. However, any deviation from the method can invalidate the results. For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for antimicrobial susceptibility testing. Antimicrobial agents suggested for use in antimicrobial susceptibility testing of *S.* Typhi are listed in Table 13.

Special considerations for antimicrobial susceptibility testing of S. *Typhi*

As previously mentioned, testing some bacteria against certain antimicrobial agents may yield misleading results because these in vitro results do not necessarily correlate with in vivo activity. *Salmonella* (including ser. Typhi) isolates, for instance, are usually susceptible to aminoglycosides (*e.g.*, gentamicin, kanamycin) and first- and second-generation cephalosporins using the disk diffusion test, but treatment with these drugs is often not effective [NCCLS 2002].

[31] Formerly known as the "National Committee for Clinical Laboratory Standards," NCCLS is now known solely by its acronym.

TABLE 13: Antimicrobial agents suggested for use in antimicrobial susceptibility testing of ***Salmonella*** ser. Typhi

Antimicrobial agents for susceptibility testing of Salmonella serotype Typhi
Ampicillin
Chloramphenicol
Trimethoprim-sulfamethoxazole (cotrimoxazole)
Nalidixic acid*

**If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.*

It is also worth noting that **sometimes the result of one antimicrobial susceptibility test will indicate the need for additional tests** to confirm an expected result. For example, when an isolate of *S.* Typhi is resistant to nalidixic acid, it will usually exhibit reduced susceptibility to ciprofloxacin; this scenario may translate in the clinical setting to need for a longer course of treatment. Isolates exhibiting resistance to nalidixic acid should be tested for susceptibility to ciprofloxacin.

Agar disk diffusion testing of *S.* Typhi

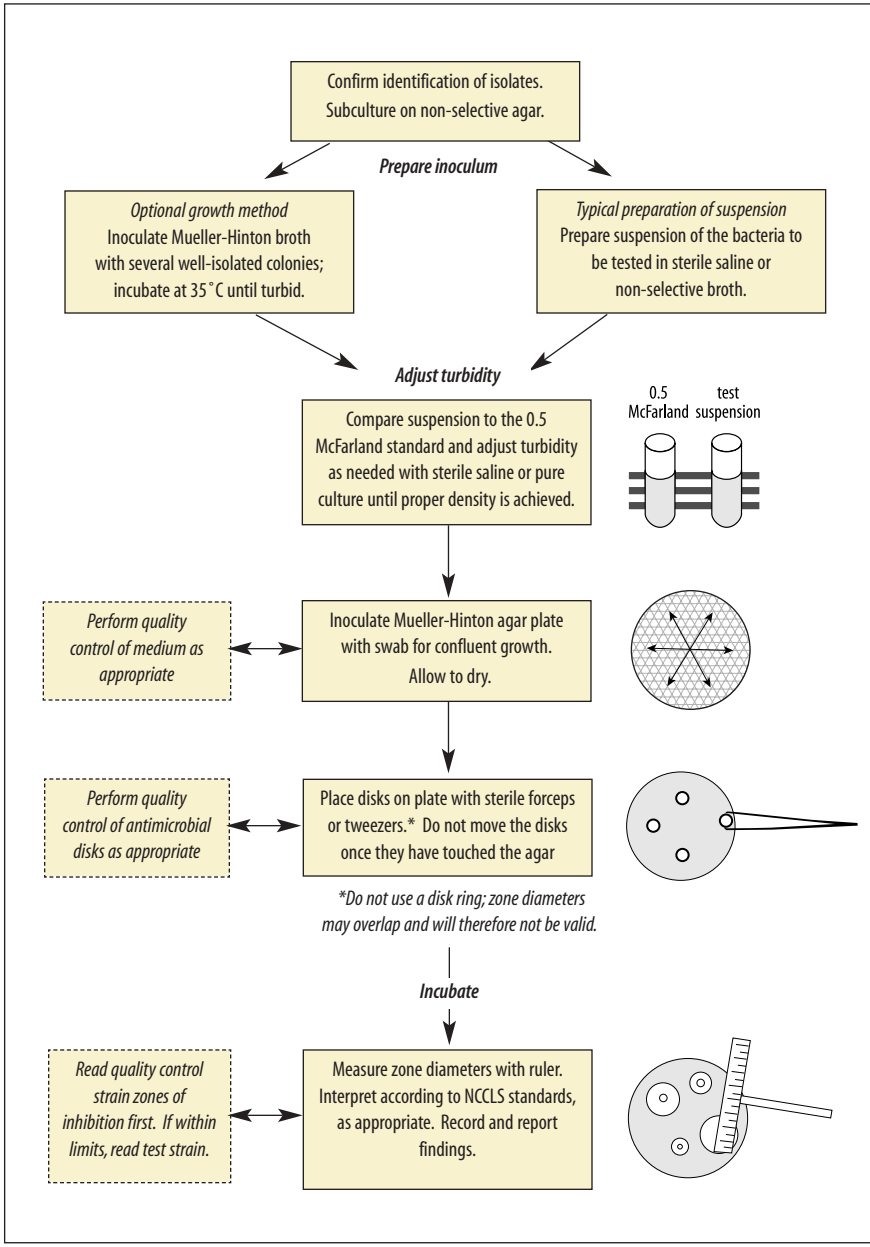
Mueller-Hinton agar medium is the only antimicrobial susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar should always be used for disk diffusion susceptibility testing according to NCCLS and international guidelines. Because the way in which Mueller-Hinton is prepared can affect disk diffusion test results, this medium should be prepared strictly according to the methods and quality control instructions presented in Appendix 2. A summary of the disk diffusion method of antimicrobial susceptibility testing is presented in Figure 33.

A 0.5 McFarland turbidity standard should be prepared and quality controlled prior to beginning antimicrobial susceptibility testing (see Appendix 2, Figure 50). If tightly sealed to prevent evaporation and stored in the dark, the turbidity standard can be stored for up to 6 months. The 0.5 McFarland turbidity standard is used to adjust the turbidity of the inoculum for the antimicrobial susceptibility test.

Preparation of inoculum

Each culture to be tested should be streaked onto a non-inhibitory agar medium (*e.g.*, blood agar, brain heart infusion agar, or tryptone soy agar [TSA]) to obtain isolated colonies. After incubation at 35˚C overnight, select four or five well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline or nonselective broth (*e.g.*, Mueller-Hinton broth, heart infusion broth, or tryptone soy broth [TSB]) and vortex thoroughly. The bacterial

FIGURE 33: Flowchart of the general procedure for antimicrobial susceptibility testing by disk diffusion



suspension should then be compared to the 0.5 McFarland turbidity standard. This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (see Appendix 2, Figures 51 and 52). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the 0.5 McFarland turbidity standard, the turbidity can be reduced by adding sterile saline or broth or increased by adding more bacterial growth.

Alternatively, the growth method may be used to prepare the inoculum. Pick four or five colonies from overnight growth on agar and inoculate them into broth (Mueller-Hinton broth, heart infusion broth, or TSB). Incubate the broth at 35˚C until turbid (usually 16–24 hours), and then adjust the turbidity to the proper density.

Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 34). Finally, swab around the entire edge of the agar surface.

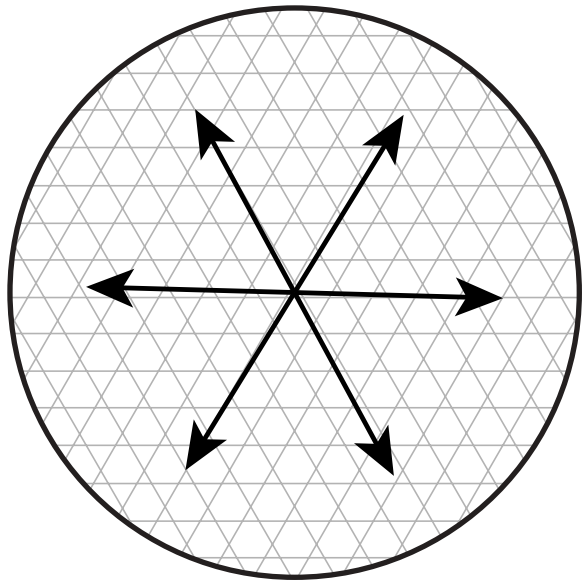
If the bacterial colonies used to prepare the suspension are picked off a plate containing mixed growth (*i.e.*, if isolated colonies are picked from a plate that does not contain pure culture, as may occur when working with cultures from stool specimens), laboratorians may choose to prepare a purity plate to ensure the suspension used for antimicrobial susceptibility testing is pure. To prepare the purity plate, after inoculating the Mueller-Hinton agar plate for confluent growth, label (a portion of) a separate TSA plate (or other non-selective medium) and **use the same swab** of suspension with which the Mueller-Hinton was inoculated to streak for isolation; do not place the swab back into the suspension. Several inocula can be streaked on different sections of a properly labeled purity plate, but the streaks **must not overlap.**

Quality control

To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. (ATCC 25922 is the *E. coli* control strain used when testing *S.* Typhi and other *Enterobacteriaceae.*) Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits; Table 14 includes the diameters of the zones of inhibition for ATCC 25922. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be a

FIGURE 34: Inoculation of Mueller-Hinton medium for antimicrobial susceptibility tests



Inoculate a Mueller-Hinton plate by dipping a sterile swab into the standardized inoculum one time and then swabbing the entire surface of the medium three times, rotating the plate after each application to ensure an even inoculum for confluent growth.

source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected. For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks, even when the isolates being tested are susceptible.

TABLE 14: Inhibition zone diameter size interpretive standards for ***Enterobacteriaceae*** (for selected antimicrobial disks appropriate for ***Salmonella*** ser. Typhi)

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml)			NCCLS QC strain
		Susceptible	Intermediate	Resistant	***E. coli*** ATCC 25922
Ampicillin	10 µg	≥ 17 mm *(≤ 8 µg/ml)*	14 – 16 mm *(16 µg/ml)*	≤ 13 mm *(≥ 32 µg/ml)*	16 – 22 mm *(2–8 µg/ml)*
Chloramphenicol	30 µg	≥ 18 mm *(≤ 8 µg/ml)*	13 – 17 mm *(16 µg/ml)*	≤ 12 mm *(≥ 32 µg/ml)*	21 – 27 mm *(2–8 µg/ml)*
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 µg	≥ 16 mm *(≤ 2/38 µg/ml)*	11 – 15 mm *(4/76 µg/ml)*	≤ 10 mm *(≥ 8/152 µg/ml)*	23 – 29 mm *(≤ 0.5/9.5 µg/ml)*
Nalidixic acid	30 µg	≥ 19 mm *(≤ 8 µg/ml)*	14 – 18 mm *(16 µg/ml)*	≤ 13 mm *(≥ 32 µg/ml)*	22 – 28 mm *(1–4 µg/ml)*
Ciprofloxacin	5 µg	≥ 21 mm *(≤ 1 µg/ml)*	16 – 20 mm *(2 µg/ml)*	≤ 15 mm *(≥ 4 mg/ml)*	30 – 40 mm *(0.004–0.016 µg/ml)*

Source: NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.

If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease; this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

Antimicrobial disks

The working supply of antimicrobial disks should be stored in a refrigerator (4˚C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate; this reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before use.

Apply the antimicrobial disks to the plates as soon as possible, but no longer than 15 minutes after inoculation. The plate surface should be dry, with no liquid remaining. Place the disks individually with sterile forceps or with mechanical dispensing apparatus, and then gently press down onto the agar. In general, no more than 12 disks should be placed on a 150-mm plate and **no more than four disks should be placed on a 100-mm plate** to prevent overlapping of the zones of inhibition and possible resultant error in measurement. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.**

Recording and interpreting results

After the disks are placed on the plate, invert the plate and incubate at 35°C for 16–18 hours; if a purity plate was prepared, incubate it under the same conditions. After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) (Figure 28) and record it in millimeters. (A sample worksheet is included in Figure 35.) The measurements can be made with calipers or a ruler on the undersurface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone; in this instance, slight growth (approximately 80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. For *S.* Typhi, zones of growth inhibition should be compared with the zone-size interpretative table for *Enterobacteriaceae* (Table 14), and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. Measure the distance from the inner-most colonies (*i.e.*, those closest to the disk) to the center of the antimicrobial disk, and double this measurement to obtain the diameter; record the measurement and interpretation of antimicrobial susceptibility (Figure 35). If there is both an inner- and outer zone of inhibition of growth around the antimicrobial disk:

a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)

b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).

c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

The presence of colonies within a zone of inhibition may predict eventual resistance to that antimicrobial agent.

Data for decision-making

Once the laboratory has assessed the identity and antimicrobial susceptibility patterns of *S.* Typhi isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

FIGURE 35: Sample form for recording antimicrobial susceptibility test results for ***Salmonella*** ser. Typhi

Date of Testing: _____/_____/_____

*Test performed by:*_____

Interpretation of susceptibility: *S* = susceptible *I* = intermediate *R* = resistant

Specimen number	Ampicillin	Chloramphenicol	Trimethoprim-sulfamethoxazole	Nalidixic acid [a]	*(other drug)* _____
	mm S I R	mm S I R	mm S I R	mm S I R	mm S I R
	mm S I R	mm S I R	mm S I R	mm S I R	mm S I R
	mm S I R	mm S I R	mm S I R	mm S I R	mm S I R
	mm S I R	mm S I R	mm S I R	mm S I R	mm S I R
E. coli ATCC 25922 (NCCLS QC strain) *QC in range?* →	mm Yes No	mm Yes No	mm Yes No	mm Yes No	mm Yes No

[a] If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.

Reviewed by: _____ ***Date of Report:*** ___/___/_____

Note: After 16-18 hours incubation, check the results for the NCCLS-recommended quality control (QC) strain E. coli ATCC 25922 against the acceptable range of inhibition zone diameters and then record disk diffusion results (mm). (Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 14.)

- Immunization with available typhoid fever vaccines should be considered only for high-risk populations where epidemic or high endemic rates of multi-drug resistant *S.* Typhi infections are a major cause of morbidity and mortality, **and** where vaccine effectiveness can be formally evaluated.

Consideration of such factors when making decisions based on data will help public health officials meet needs in a manner appropriate to the local situation and antimicrobial susceptibility profile.

CHAPTER VIII

Shigella

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

The genus *Shigella* is divided into four subgroups: *Shigella dysenteriae* (Subgroup A), *Shigella flexneri* (Subgroup B), *Shigella boydii* (Subgroup C), and *Shigella sonnei* (Subgroup D). Each of these subgroups, with the exception of *S. sonnei*, has several serotypes (Table 15). In general, *S. sonnei* is more common in developed countries and *S. flexneri* and *S. dysenteriae* serotype 1 occur more frequently in developing countries. The proportions of each subgroup varies from country to country, though epidemic dysentery in developing countries is usually caused by *S. dysenteriae* 1, an unusually virulent pathogen. The hallmark of infection with *Shigella* is diarrhea with blood, often termed "dysentery." However, in almost half of cases, *Shigella* causes acute non-bloody diarrheas that cannot be distinguished clinically from diarrhea caused by other enteric pathogens. Severity of symptoms appears to be dose-related.

Shigella dysenteriae serotype 1 differs from other *Shigella* in several ways:

- Only *S. dysenteriae* 1 causes large and prolonged epidemics of dysentery.
- Infection with *S. dysenteriae* 1 causes more severe, more prolonged, and more frequently fatal illness than does infection with other *Shigella.*
- Antimicrobial resistance develops more quickly and occurs more frequently in *S. dysenteriae* 1 than in other *Shigella* groups.

This section of the laboratory manual focuses on the isolation, identification, and antimicrobial susceptibility testing of *Shigella.*

Identification of *Shigella*

Methods for the collection and transport of fecal specimens and the primary isolation and presumptive identification on selective agar are included in

Appendices 9 and 10. Suspect *Shigella* isolates should be subcultured to a nonselective medium (*e.g.*, Kligler iron agar [KIA] or triple sugar iron agar [TSI]) in preparation for identification by slide serology and biochemical tests. Figure 36 presents a flowchart for the isolation and identification of an isolate as *Shigella*, and Figure 37 provides a sample worksheet which can be used to record test results.

Biochemical screening tests

Identification of *Shigella* subgroups involves both biochemical and serologic testing. The use of biochemical screening media is usually advisable to avoid wasting antisera. For most laboratories, KIA or TSI will be the single most helpful medium for screening suspected *Shigella* isolates. If an additional test is desired, motility medium can be used to screen isolates before serologic testing is performed.

Kligler iron agar and triple sugar iron agar

To obtain true reactions in KIA, TSI, or other biochemical tests, a pure culture must be used to inoculate the medium. Carefully select at least one of each type of well-isolated colony on each type of plate that was streaked for isolation (*i.e.*, if suspect lactose-nonfermenting colonies that differ in macroscopic appearance are present, a separate test should be run for each.) Using an inoculating needle, lightly touch only the center of the colony. **Do not take the whole colony or go through the colony and touch the surface of the plate** because doing so can pick up contaminants that may be on the surface of the agar. If there is doubt that a particular colony is sufficiently isolated from surrounding colonies, purify the suspicious colony by streaking on another agar plate; afterwards, the KIA slant or TSI slant may be inoculated. Only one colony should be inoculated into each test medium.

KIA and TSI tubes are inoculated by stabbing the butt and streaking the surface of the slant. After incubation for 18–24 hours at 35°–37°C, the slants are observed for reactions typical of *Shigella*. When incubating most biochemicals, caps should be loosened before placement in the incubator. This is particularly important when using KIA and TSI. **If the caps are too tight and anaerobic conditions exist in KIA or TSI, the characteristic reactions of *Shigella* may not occur and a misleading result could be exhibited.** In addition, the KIA and TSI tubes must be prepared so that the tubes have a deep butt (*i.e.*, approximately 3.5 cm) and a long slant (*i.e.*, approximately 2.5 cm). *Shigella* characteristically produces an alkaline (red) slant and an acid (yellow) butt, little or no gas, and no H_2S (see Table 15 and Figure 38). A few strains of *S. flexneri* serotype 6 and very rare strains of *S. boydii* produce gas in KIA or TSI.

FIGURE 36: Flowchart for the isolation and identification of ***Shigella***

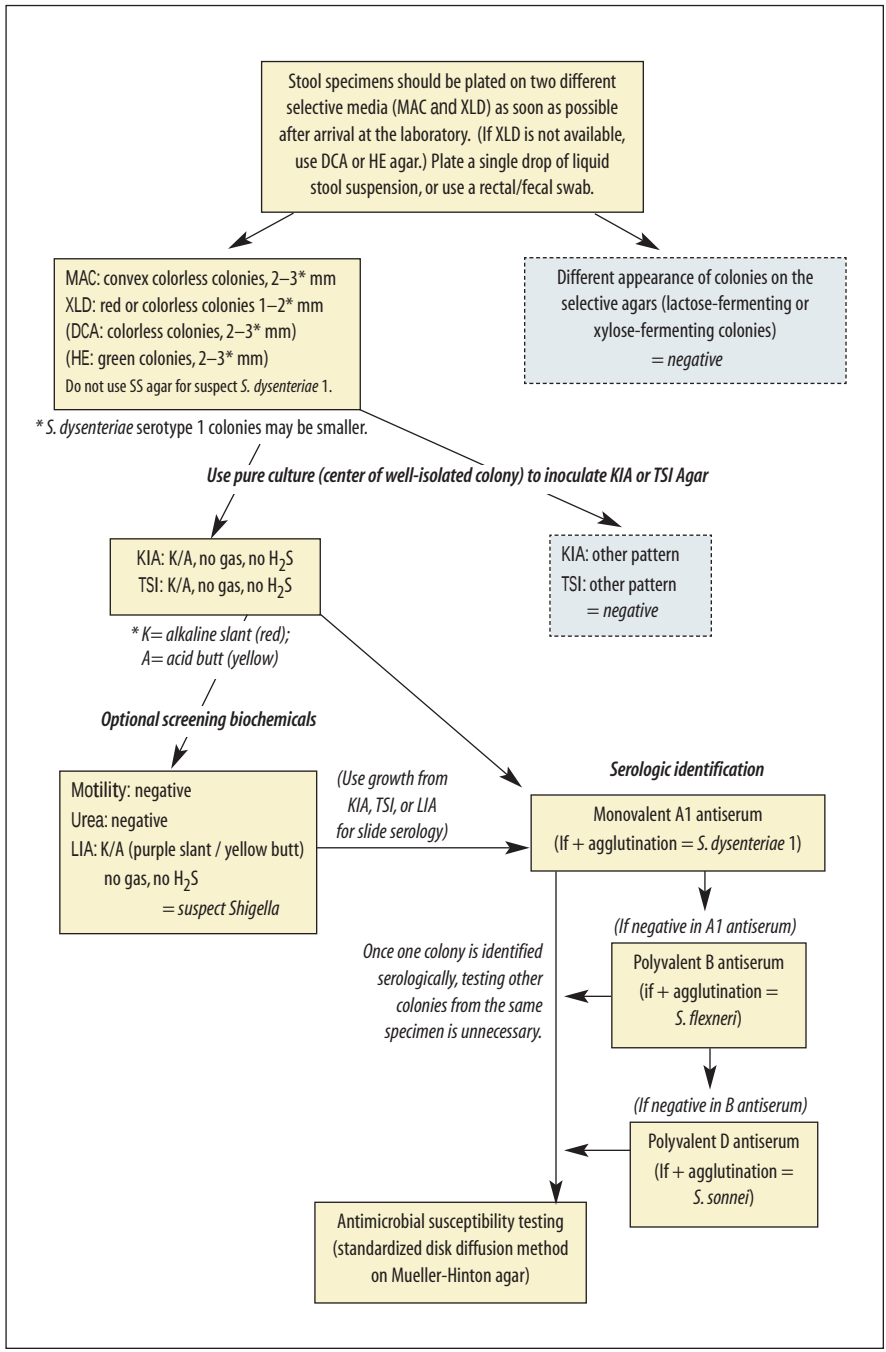


FIGURE 37: Sample worksheet for ***Shigella*** test results

Specimen number	Medium	XYL/ LAC-[a]	XYL/ LAC+[a]	Colony[b]	KIA / TSI	OPTIONAL			SLIDE SEROLOGY [c]			Identification
						Motility	Urea	LIA	A1	B	D	
				X1								
	XLD			X2								
				X3								
				M1								
	MAC			M2								
				M3								
				X1								
	XLD			X2								
				X3								
				M1								
	MAC			M2								
				M3								
				X1								
	XLD			X2								
				X3								
				M1								
	MAC			M2								
				M3								

[a] XYL/LAC–: Xylose or Lactose -negative colonies
XYL/LAC+ : Xylose or Lactose -positive colonies

[b] Identification of only one colony from each suspect case must be confirmed as *Shigella*.

[c] A1 = Monovalent antiserum for *Shigella dysenteriae* (Serogroup A) serotype 1
B = Polyvalent antiserum for *Shigella flexneri* (Serogroup B)
D = Polyvalent antiserum for *Shigella sonnei* (Serogroup D)

TABLE 15: Reactions of ***Shigella*** in screening biochemicals

Screening medium	***Shigella*** reaction	Figure number
Kligler iron agar (KIA)	K/A, no gas produced (red slant/yellow butt) [a]	*Figure 38*
Triple sugar iron agar (TSI)	K/A, no gas produced (red slant/yellow butt) [a]	~
H_2S (on KIA or TSI)	Negative *(positive reaction would be blackened medium)*	~
Motility	Negative	*Figure 39*
Urea	Negative	*Figure 40*
Indole	Positive or Negative	~
Lysine iron agar (LIA)	K/A (purple slant/yellow butt) [b]	*Figure 41*

[a] K = alkaline (red); A = acid (yellow). Some strains of S. flexneri serotype 6 and S. boydii produce gas from glucose.

[b] K = alkaline (purple); A = acid (yellow). An alkaline reaction in the butt of the medium indicates that lysine was decarboxylated; an acid reaction in the butt indicates that lysine was not decarboxylated.

Motility agar

Motility agar should be inoculated with a straight inoculating needle, making a single stab about 1–2 cm down into the medium. The surface of the motility agar should be dry when used: moisture can cause a non-motile organism to grow down the sides of the agar, creating a haze of growth and appearing to be motile. Motility agar may be inoculated with growth from a KIA or TSI that shows a reaction typical of *Shigella*. Alternatively, motility agar can be inoculated at the same time as the KIA or TSI slant by using the same inoculating needle without touching the colony again. (When motility agar is to be inoculated at the same time as KIA or TSI, use the same colony to first inoculate the motility agar and then to inoculate the KIA or TSI by stabbing the butt and then streaking the surface of the slant. **Do not select a second colony to inoculate the KIA or TSI after the motility agar has been inoculated because it may represent a different organism.**

Examine after overnight incubation at 35°–37°C. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 39). Non-motile organisms do not grow out from the line of inoculation. Motility reactions may be difficult for inexperienced laboratorians to read; therefore, reactions should be compared with positive and negative control strains. *Shigella* are always non-motile (Table 15).

Sulfide-indole-motility medium is a combination medium that is commercially available in dehydrated form (see Appendix 2, "Media, Reagents, and Quality Control"). It can be used in place of motility medium.

Additional biochemical screening tests

Other biochemical tests (*e.g.*, urea medium and lysine iron agar [LIA]) may be used for additional screening of isolates before testing with antisera (Table 15).

FIGURE 38: Reaction typical of ***Shigella*** in Kligler iron agar (KIA): alkaline slant and acid butt

FIGURE 39: Motility medium showing a non-motile organism in the left tube and a motile organism (seen as clouding) in the right tube.

FIGURE 40: Reactions in urea medium

A pink color develops in a positive urease reaction (*tube on left*)

The value of these other tests should be assessed before they are used routinely; rationale for performing each test is included along with the following methods. These media, their preparation, and suggested quality control strains are described in Appendix 2.

Urea medium

Urea medium screens out urease-producing organisms (*e.g.*, *Klebsiella* and *Proteus*). Urea agar is inoculated heavily over the entire surface of the slant. Loosen caps before incubating overnight at 35°–37°C. **Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color** (Figure 40). Urease-negative organisms do not change the color of the medium, which is a pale yellowish-pink. *Shigella* are always urease-negative.

Lysine iron agar

LIA is helpful for screening out *Hafnia* spp. and certain *E. coli, Proteus*, and *Providencia* strains. LIA should be inoculated by stabbing the butt and streaking the slant. After incubation for 18–24 hours at 35°–37°C, organisms that produce lysine decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the medium and also on the slant (Figure 41). A blackening of the medium

indicates H_2S production. Organisms lacking lysine decarboxylase produce an alkaline slant (purple) and an acid butt (yellow), no gas, and no H_2S. *Proteus* and *Providencia* species will often produce a red slant caused by deamination of the lysine. Lysine iron agar must be prepared so that the tubes have a deep butt (*i.e.*, approximately 3.5 cm). *Shigella* are lysine-negative and characteristically produce an alkaline (purple) slant, an acid (yellow) butt, no gas, and no H_2S in LIA.

Serologic identification of *Shigella*

Serologic testing is needed for the identification of *Shigella* isolates. Serologic identification of *Shigella* is performed typically by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed, in some cases, by testing with monovalent antisera for specific serotype identification. Monovalent antiserum to *S. dysenteriae* 1 is required to identify this serotype, which is the most frequent cause of severe epidemic dysentery (Table 16). Once one colony from a plate has been identified as *Shigella*, no further colonies from the same specimen need to be tested.

Laboratorians should be aware that some *Shigella* commercial antiserum is labeled or packaged differently; that is, two packages with different names may contain the same antisera. For example, *Shigella* polyvalent A, which includes antisera to serotypes 1 through 7, may also be labeled polyvalent A1. Further, monovalent antiserum may be labeled such that it could be confused with polyvalent antiserum; for example, monovalent antiserum to *S. dysenteriae* 1 may be labeled "*Shigella* A1" instead of "*S. dysenteriae* serotype 1". (Table 16 can serve as a useful guide for referencing which subgroups and serotypes are associated with what *Shigella* nomenclature designation.) When using newly purchased antisera, the laboratorian should read the package insert or check with the manufacturer if the label is not self-explanatory. All lots of antisera should undergo quality control testing before use (Appendix 2).

Slide agglutination

Because *S. dysenteriae* 1 is the most common agent of epidemic dysentery (followed by *S. flexneri* and *S. sonnei*), isolates that react typically in the screening biochemicals should be screened first with monovalent A1 antiserum, then with polyvalent B antiserum, and finally in polyvalent D antiserum. When a positive agglutination reaction is obtained in one of the antisera, the *Shigella* subgroup is identified, and no further testing with antisera needs to be conducted. (Because subgroup C, *S. boydii*, is so rare it is not cost-effective to perform routine screens for it.)

a) Agglutination tests may be performed in a Petri dish or on a clean glass slide. Divide the slide into test sections with a wax pencil and place one small drop of physiological saline in each test section on the slide.

FIGURE 41: Reactions in lysine iron agar (LIA) medium

Organisms positive for lysine decarboxylase produce a purple color throughout the LIA medium *(tube on the right*). Lysine-negative organisms produce a yellow color (acid) in the butt portion of the tube (*tube on left*).

TABLE 16: Subgroup and serotype designations of ***Shigella***

Shigella (common name)	Subgroup designation (polyvalent antisera)	Serotypes (monovalent antisera)	(Label on commercial antiserum may also say)
S. dysenteriae	Group A	1 – 15[a,b]	(A1, A2, A3, . . . , A13)
S. flexneri	Group B	1 – 6, X, Y	(B1, B2, B3, B4, B5, B6)
S. boydii[c]	Group C	1 – 19[b]	(C1, C2, C3, . . . , C18)
S. sonnei	Group D	1	(D1)

[a] Detection of *S. dysenteriae* 1 is of particular importance since it is unusually virulent and causes endemic or epidemic dysentery with high death rates. Monovalent antiserum (absorbed) is required to identify *S. dysenteriae* 1.

[b] Additional provisional serotypes have been reported, but antisera to these new serotypes were not commercially available at the time this manual was printed.

[c] Because *S. boydii* is so rare it is not cost-effective to perform routine screens for it.

b) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, heart-infusion agar (HIA), or other **non-selective** agar medium. (Serologic testing should not be done on growth from selective media such as MacConkey or XLD agar because selective media may yield false-negative serologic results.) Emulsify the growth in each drop of physiological saline on the slide and mix thoroughly to create a moderately milky suspension.

c) Add a small drop of antiserum to one of the suspensions; the second suspension serves as the control. To conserve antiserum, volumes as small as 10 µl can be used; a bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32). Usually approximately equal volumes of antiserum and growth suspension are mixed, although the volume of suspension may be as much as double the volume of the antiserum.

d) Mix the suspension and antiserum well and then tilt the slide back and forth to observe for autoagglutination (Figure 2). The agglutination is more visible if the slide is observed under a bright light and over a black background. If the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 42). Examine the saline suspension carefully to ensure that it is even and does not show clumping resulting from autoagglutination. If autoagglutination occurs, the culture is termed "rough" and cannot be serotyped.

Cultures that react serologically and show no conflicting results in the biochemical screening tests are reported as positive for *Shigella*.

Antimicrobial susceptibility testing of *Shigella*

As antimicrobial resistance increases in many parts of the world, monitoring the antimicrobial susceptibility of *Shigella* becomes increasingly important. The disk

FIGURE 42: Serologic identification: agglutination reactions of ***Shigella***

Shigella antiserum will agglutinate strains of the same subgroup or serotype (*right*); in contrast, the *Shigella* suspension on the left did not agglutinate when mixed with saline.

diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),[32] and if performed precisely according to the protocol below, will provide data that can reliably predict the *in vivo* effectiveness of the drug in question. However, **any deviation from the method may invalidate the antimicrobial susceptibility test results.** For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for antimicrobial susceptibility testing.

Specific methods for determination of antimicrobial susceptibility of *Shigella* are presented in this chapter; however, there are some general guidelines that must first be considered before proceeding: test isolates from the beginning of an outbreak; test appropriate antimicrobial agents; provide timely feedback to public health officials; and, periodically monitor the epidemic for shifts in antimicrobial susceptibility patterns.

[32] Formerly known as the "National Committee for Clinical Laboratory Standards," NCCLS is now known solely by its acronym.

- *Test the isolates from the beginning of an outbreak*
  Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic. That number will provide sufficient information to develop an antimicrobial treatment policy for the organism. After that, the laboratory should conduct periodic surveys to detect any changes in antimicrobial susceptibility patterns. (World Health Organization [WHO] surveillance manuals can be useful guides for survey design.)

- *Test appropriate antimicrobial agents*
  The laboratory should routinely test only those antimicrobial agents that are available in the country or antimicrobial agents that are recommended by WHO as efficacious in the treatment of shigellosis (Table 17). In addition, if all isolates are resistant to a particular antimicrobial agent (*e.g.*, to ampicillin) during the first round of testing, testing against those agents during future surveys of the outbreak strain is probably not warranted (although testing of isolates may still be performed once or twice a year to confirm resistance). Sending 10 to 20 of the initial isolates to an international reference laboratory can be useful for confirmatory identification of the strain and antimicrobial susceptibility pattern. Guidelines for the packing and shipping of etiologic agents are included in Appendix 12.

- *Provide timely feedback to public health officials*
  Once the organisms are isolated and the antimicrobial susceptibility patterns determined, these results should be transmitted as quickly as possible to the national epidemiologist and to other public health officials. The data can then be used to make rational choices for antimicrobial treatment policy.

- *Monitor for changes in antimicrobial susceptibility*
  As a dysentery epidemic progresses, periodic surveys of 30 to 50 isolates of the epidemic organism should be carried out to detect any changes in the antimicrobial susceptibility pattern of the organism causing the epidemic. These surveys should be conducted every 2–6 months, depending on conditions and resources. Any changes should be reported to the national epidemiologist and to other public health officials so that the antimicrobial treatment policy can be modified, if necessary. If any major changes are noted, it is useful to send isolates to an international reference laboratory for confirmation.

Antimicrobial agents for treatment and testing of *Shigella*

The following antimicrobial agents are recommended by the WHO for treatment of *Shigella* infections: ampicillin, ciprofloxacin, norfloxacin, enoxacin, nalidixic acid, pivmecillinam, and trimethoprim-sulfamethoxazole (often referred to as cotrimoxazole).

Antimicrobial agents suggested for use in susceptibility testing of *Shigella* are listed in Table 17; these WHO recommendations are current as of the date of publication of this document.

Testing *Shigella* against certain drugs may yield misleading results when *in vitro* results do not correlate with in vivo activity. *Shigella* isolates, for instance, are usually susceptible to aminoglycosides (*e.g.*, gentamicin, kanamycin) and first- and second-generation cephalosporins in the disk diffusion test, but treatment with these drugs is often not effective [NCCLS 2002].

The selection of antimicrobial treatment should be based on the results of recent antimicrobial susceptibility testing of *Shigella* strains obtained from the same region (or from nearby areas if *Shigella* is new to the area). Unfortunately, resistance of *Shigella* to ampicillin and trimethoprim-sulfamethoxazole has become widespread. Nalidixic acid, formerly used as a "backup" drug to treat resistant shigellosis, is now the drug of choice in most areas, but resistance to it has appeared in many places. When resistant to nalidixic acid, *Shigella* should be tested with ciprofloxacin; strains resistant to nalidixic acid often exhibit reduced susceptibility to ciprofloxacin. Pivmecillinam (*i.e.*, amdinocillin pivoxil) is still effective for most strains of *Shigella* but may not be readily available. Fluoroquinolones (*e.g.*, ciprofloxacin, norfloxacin, and enoxacin) are often costly and may not be readily available; fluoroquinolones should be considered only if *Shigella* isolates are resistant to nalidixic acid.

As of the publication of this document (2002), *Shigella* strains are often resistant to ampicillin, trimethoprim-sulfamethoxazole, metronidazole, streptomycin, tetracycline, chloramphenicol, and sulfonamides. In addition, although *Shigella* may be susceptible to some antimicrobial agents *in vitro*, the drug may have no documented efficacy *in vivo*. Examples of such agents are nitrofurans (*e.g.*, nitrofurantoin, furazolidone), aminoglycosides (*e.g.*, gentamicin, kanamycin), first- and second-generation cephalosporins (*e.g.*, cephalexin, cefamandol), and amoxicillin.

TABLE 17: Antimicrobial agents suggested for use in antimicrobial susceptibility testing of ***Shigella***

Antimicrobial agents for susceptibility testing of ***Shigella***
Trimethoprim-sulfamethoxazole (cotrimoxazole)
Chloramphenicol
Ampicillin
Nalidixic acid *

**If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.*

Procedure for agar disk diffusion antimicrobial susceptibility testing of *Shigella*

The disk diffusion method of antimicrobial susceptibility testing is similar to that described in the S. Typhi chapter, and is summarized in Figure 33. Laboratory diagnostic supplies required for *Shigella* disk diffusion testing are listed in Appendix 9. This section provides seven steps for antimicrobial susceptibility testing of *Shigella* by the disk diffusion method.

1. ***Mueller-Hinton antimicrobial susceptibility test agar***
   Mueller-Hinton agar medium is the only antimicrobial susceptibility test medium that has been validated by NCCLS. **Mueller-Hinton agar, poured to a uniform depth of 3–4mm, should always be used for disk diffusion antimicrobial susceptibility testing,** according to NCCLS and international guidelines. Because the way in which Mueller-Hinton is prepared can affect disk diffusion test results, this medium should be prepared strictly according to the methods and quality control instructions presented in Appendix 2.

2. ***McFarland turbidity standard***
   A 0.5 McFarland turbidity standard should be prepared and quality controlled prior to beginning antimicrobial susceptibility testing (Appendix 2, Figure 50). If tightly sealed to prevent evaporation and stored in the dark, the turbidity standard can be stored for up to 6 months. The 0.5 McFarland turbidity standard is used to adjust the turbidity of the inoculum for the antimicrobial susceptibility test.

3. ***Preparation of inoculum***
   Each culture to be tested should be streaked onto a non-inhibitory agar medium (*e.g.*, blood agar, brain heart infusion agar, or tryptone soy agar [TSA]) to obtain isolated colonies. After incubation at 35°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline or nonselective broth (*e.g.*, Mueller-Hinton broth, heart infusion broth, or tryptone soy broth [TSB]) and vortex thoroughly. **The bacterial suspension should then be compared to the 0.5 McFarland turbidity standard.** This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (Figures 51 and 52 in the McFarland turbidity standard section of Appendix 2). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the 0.5 McFarland turbidity standard, the turbidity can be reduced by adding sterile saline or broth, or increased by adding more bacterial growth.

   Alternatively, the growth method may be used to prepare the inoculum. Pick four or five colonies from overnight growth on agar and inoculate them into broth (Mueller-Hinton broth, heart infusion broth, or TSB). Incubate the broth at 35°C until turbid (usually 16–24 hours), and then adjust the turbidity to the proper density.

4. ***Inoculation procedure***
   Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 34). Finally, swab around the entire edge of the agar surface.

5. ***Antimicrobial disks***
   The working supply of antimicrobial disks should be stored in the refrigerator (at 4°C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate; this reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before using.

   Apply the antimicrobial disks to the plates as soon as possible after the plate is dry, but no longer than 15 minutes after inoculation. Place the disks individually with sterile forceps or with a mechanical dispensing apparatus, equidistant from each other, and then gently press down onto the agar. In general, **no more than 12 disks are placed on a 150-mm plate and no more than four disks are placed on a 100-mm plate** to prevent overlapping of the zones of inhibition and possible resultant error in measurement. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.** After the disks are placed on the plate, invert the plate and incubate at 35°C for 16–18 hours.

6. ***Recording and interpreting results***
   After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) (Figure 43) and record it in millimeters. (A sample worksheet is provided in Figure 44.) The measurements can be made with calipers or a ruler on the undersurface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (approximately 80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. The zones of growth inhibition should be compared with the zone-size interpretative table (Table 18), and recorded as susceptible, intermediate, or resistant to each drug tested.

   Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. Measure the distance from the inner-most colonies (*i.e.*, those closest to the disk) to the center of the antimicrobial disk, and double this measurement to obtain the diameter; record the measurement and interpretation of antimicrobial susceptibility (Figure 44). If there is

both an inner- and outer zone of inhibition of growth around the antimicrobial disk:

a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)

b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).

c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

The presence of colonies within a zone of inhibition may predict eventual resistance to that antimicrobial agent.

7. Quality control

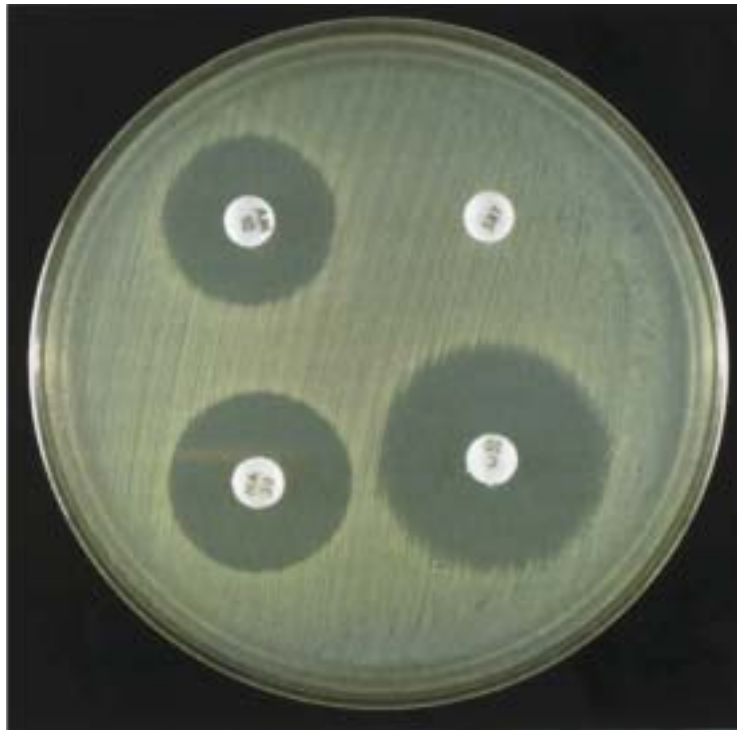
To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. (ATCC 25922 is the *E. coli* control strain used when testing *Enterobacteriaceae* [*e.g.*, *Shigella*, *Salmonella*, *Escherichia*, *Klebsiella*] and *V. cholerae.*) Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits; Table 18 includes the diameters of the zones of inhibition for ATCC 25922. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

TABLE 18: Zone size interpretive standards for ***Enterobacteriaceae*** for selected antimicrobial disks appropriate for the testing of ***Shigella***

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint(µg/ml)			NCCLS QC strain
		Susceptible	Intermediate	Resistant	***E. coli*** ATCC 25922
Ampicillin	10 µg	≥ 17 mm *(≤ 8 µg/ml)*	14 – 16 mm *(16 µg/ml)*	≤ 13 mm *(≥ 32 µg/ml)*	16 – 22 mm *(2–8 µg/ml)*
Chloramphenicol	30 µg	≥ 18 mm *(≤ 8 µg/ml)*	13 – 17 mm *(16 µg/ml)*	≤ 12 mm *(≥ 32 µg/ml)*	21 – 27 mm *(2–8 µg/ml)*
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 µg	≥ 16 mm *(≤ 2/38 µg/ml)*	11 – 15 mm *(4/76 µg/ml)*	≤ 10 mm *(≥ 8/152 µg/ml)*	23 – 29 mm *(≤ 0.5/9.5 µg/ml)*
Nalidixic acid	30 µg	≥ 19 mm *(≤ 8 µg/ml)*	14 – 18 mm *(16 µg/ml)*	≤ 13 mm *(≥ 32 µg/ml)*	22 – 28 mm *(1–4 µg/ml)*
Ciprofloxacin	5 µg	≥ 21 mm *(≤ 1 µg/ml)*	16 – 20 mm *(2 µg/ml)*	≤ 15 mm *[≥ 4 µg/ml)*	30 – 40 mm *(0.004–0.016 µg/ml)*

Source: NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.

FIGURE 43: Sample results of the disk diffusion assay



This *Shigella* isolate is resistant to trimethoprim-sulfamethoxazole (cotrimoxazole) and is growing up to the disk (SXT), the zone of which is recorded as 6 mm. In addition to the zones of inhibited growth, note how evenly distributed the growth is on the plate.

Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected. For instance, a resistant organism could appear to be susceptible if the inoculum is too light.

FIGURE 44: Sample form for recording antimicrobial susceptibility test results for *Shigella* isolates

Date of Testing: ______/______/__________

*Test performed by:*___________________________

Interpretation of susceptibility: *S* = susceptible *I* = intermediate *R* = resistant

Specimen number	Ampicillin	Chloramphenicol	Trimethoprim-sulfamethoxazole	Nalidixic acid [a]	*(other drug)* __________
	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*
	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*
	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*
	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*	mm *S I R*
E. coli ATCC 25922 (NCCLS QC strain) *QC in range?* →	mm *Yes No*	mm *Yes No*	mm *Yes No*	mm *Yes No*	mm *Yes No*

[a] If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin and will probably exhibit reduced susceptibility to ciprofloxacin.

Reviewed by: ____________ ***Date of Report:*** ____/____/________

Note: After 16–18 hours incubation, check the results for the NCCLS-recommended quality control (QC) strain E. coli ATCC 25922 against the acceptable range of inhibition zone diameters and then record disk diffusion results (mm). (Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 18.)

Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks even when the isolates being tested are susceptible.

If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease; this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

Data for decision-making: *informed epidemic response*

Once the laboratory has assessed the identity and antimicrobial susceptibility patterns of the *Shigella* isolates, the information should be reported to public health officials in a timely manner. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent chosen should be effective against at least 80% of local *Shigella* strains.
- The antimicrobial agent chosen should be able to be given by mouth.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and antimicrobial susceptibility profile.

CHAPTER IX

Vibrio cholerae

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Most *Vibrio cholerae* isolated during cholera outbreaks will be toxigenic serogroup O1 or O139. Because the cultural and biochemical characteristics of these two serogroups are identical, the isolation and identification methods described below apply to both O1 and O139. Both serogroups must be identified using O-group-specific antisera.

Isolates of *V. cholerae* serogroup O1 are classified into two biotypes, El Tor and classical, on the basis of several phenotypic characteristics. Currently, the El Tor biotype is responsible for virtually all of the cholera cases throughout the world, and classical isolates are not encountered outside of India or Bangladesh. In addition, *V. cholerae* O1 is classified into two serotypes (Inaba and Ogawa) on the basis of agglutination in antiserum. A possible third serotype, Hikojima, has been described, but it occurs only rarely. During an outbreak or epidemic, it is worth documenting the biotype and serotype of the isolate; however, it is not necessary to know this information to respond appropriately to the epidemic.

V. cholerae serogroup O139 appeared in India in late 1992. It quickly spread to Bangladesh and other Asian countries, although the rate of spread has slowed after the initial outbreaks. Through 1998, 11 countries have officially reported transmission of *V. cholerae* O139 to the World Health Organization (WHO). Imported cases have been reported from the United States and other countries. At this time, endemic *V. cholerae* O139 appears to be confined to Asia.

Fluid replacement is the cornerstone of cholera treatment, and rehydration therapy is a necessity. Antimicrobial therapy is helpful, although not essential, in treating cholera patients. Antimicrobial agents reduce the duration of illness, the volume of stool, and the duration of shedding of vibrios in the feces. When antimicrobial agents are used, it is essential to choose one to which the organism is susceptible. Antimicrobial agents recommended by WHO for treating cholera patients as of the date of publication of this document include tetracycline, doxycycline,

furazolidone, trimethoprim-sulfamethoxazole, erythromycin, or chloramphenicol. Ciprofloxacin and norfloxacin are also effective. Because antimicrobial resistance has been a growing problem in many parts of the world, the susceptibility of *V. cholerae* O1 strains to antimicrobial agents should be determined at the beginning of an epidemic and be monitored periodically. Methods for antimicrobial susceptibility testing of *V. cholerae* are addressed in this chapter of the manual, after identification. Isolation and presumptive identification of *V. cholerae* from fecal specimens are included in Appendix 10.

Public health authorities in regions that experience outbreaks of cholera may find that the manual *Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera* [CDC 1999] provides additional helpful discussions about cholera epidemiology and laboratory decision-making in resource-limited regions. The document is available from WHO in English and French; details for ordering are included in Appendix 15.

Identification of *V. cholerae*

Methods for the collection and transport of fecal specimens and the primary isolation and presumptive identification on selective agar are included in Appendices 9 and 10. Suspect *V. cholerae* isolates should be subcultured to a non-selective medium (*e.g.*, heart infusion agar [HIA] or tryptone soy agar [TSA]) in preparation for identification by slide serology and biochemical tests. *V. cholerae* requires 0.5% NaCl (*i.e.*, salt) for optimal growth on agar media; some commercially available formulations of nutrient agar do not contain salt and should not be used for culture of *V. cholerae*. In general, screening with biochemical tests prior to testing with O1 and O139 antisera is not necessary for suspected *V. cholerae* isolates from fecal specimens. However, **if the supply of O-antigen antisera is limited, biochemical tests may be useful for additional screening of isolates before testing them with antisera.** Screening tests and slide serology **must be** performed with growth from **nonselective** media. Figure 45 presents a flowchart for isolation and identification of an isolate as *V. cholerae*, and Figure 46 provides a sample worksheet that can be used to record screening test results.

Oxidase test

The oxidase test uses Kovac's reagent (a 1% [wt/vol] solution of *N, N, N', N'* –tetramethyl-ρ-phenylenediamine dihydrochloride) to detect the presence of cytochrome c in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. The oxidase test can be performed on filter paper or on a swab.

FIGURE 45: Flowchart for isolation and identification of ***Vibrio cholerae***

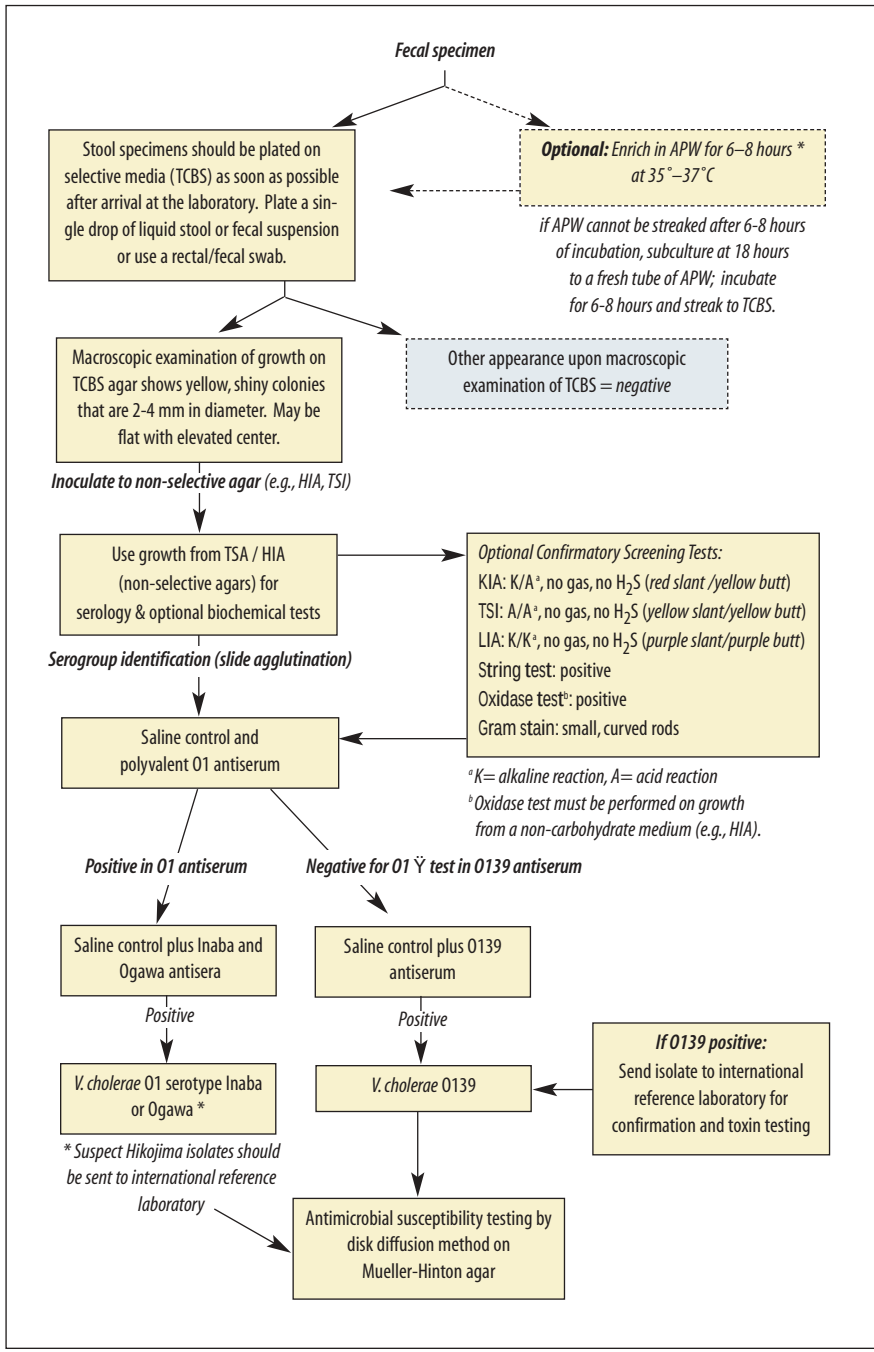


FIGURE 46: Sample worksheet for ***Vibrio cholerae*** test results

Specimen number	Medium	SUC+[b]	SUC-[c]	Colony	Optional				Slide Serology[d]				Identification
					Oxidase test	String test	Gram stain or wet mount		PV 01	Inaba	Ogawa	O139	
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS[a]			AT1									
				AT2									
				AT3									
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS[a]			AT1									
				AT2									
				AT3									
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS[a]			AT1									
				AT2									
				AT3									

It is only necessary to identify one colony **from each suspect case** as *V. cholerae*

[a] APW-TCBS : alkaline peptone water enrichment prior to inoculating TCBS
[b] SUC + : Sucrose-positive colonies
[c] SUC - : Sucrose-negative colonies

[d] PV01= polyvalent antiserum for *V. cholerae* serogroup 01
Inaba= monovalent antiserum for *V. cholerae* 01 serotype Inaba
Ogawa= monovalent antiserum for *V. cholerae* 01 serotype Ogawa
O139 = monovalent antiserum for *V. cholerae* serogroup O139

Perform the oxidase test with fresh growth from an HIA or TSA slant or any non-selective, non-carbohydrate-containing medium; **do not use growth from thiosulfate citrate bile-salts sucrose [TCBS] agar** because it may yield either false-negative or false-positive results. **Do not perform this test with a Nichrome loop,** as it may produce a false-positive reaction. Positive and negative controls should be tested at the same time as the test isolate for quality control purposes. Preparation of the oxidase reagent is described in Appendix 2.

Moistened filter paper method

a) Add two to three drops of Kovac's oxidase reagent to a piece of filter paper in a petri dish and allow it to absorb; the filter paper should be moist (but not wet) after the reagent has been absorbed.

b) Using a platinum loop, a plastic loop, a sterile swab, or a sterile wooden applicator stick or toothpick, pick a portion of the colony to be tested **from non-selective media** and rub it onto the moistened filter paper. (**Do not use a Nichrome loop.**)

c) If the isolate is *V. cholerae*, a positive (purple) reaction should occur in the region where the growth has been smeared within 10 seconds (Figure 10).

Swab method

a) Pick up suspect colonies from a non-selective culture plate or growth from a non-selective agar slant with the swab.

b) Use a Pasteur pipette to add one drop of Kovac's oxidase reagent to the swab.

c) If the isolate is *V. cholerae*, a positive (purple) reaction should occur within 10 seconds. (See Figure 20).

If an isolate has not turned purple within 10 seconds of adding the Kovac's oxidase reagent, **it is not considered oxidase-positive.** Organisms of the genera *Vibrio* (Table 19), *Neisseria, Campylobacter, Aeromonas, Plesiomonas, Pseudomonas,* and *Alcaligenes* are all oxidase-positive; all *Enterobacteriaceae* are oxidase-negative.

Additional biochemical screening tests

The string reaction, Kligler iron agar (KIA) or triple sugar iron agar (TSI), lysine iron agar (LIA), Gram stain, and wet mount for motility are other possible tests that may be used for additional screening of isolates before testing with antisera (Table 19). The value of these other tests should be assessed before they are used routinely; rationale for performing each test (*e.g.*, use of the string test to rule out *Aeromonas*) is included along with the following methods. These media, their preparation, and suggested quality control strains are described in Appendix 2.

TABLE 19: Reactions of ***Vibrio cholerae*** in screening tests

Screening test	***Vibrio cholerae*** reactions	Figure number
Oxidase test	Positive	*Figure 10* and *Figure 20*
String test	Positive	*Figure 47*
Kligler iron agar (KIA)	K/A (red slant/yellow butt)[a], no gas produced, no H_2S [18–24 hours]	*Figure 48*
Triple sugar iron agar (TSI)	A/A (yellow slant/yellow butt)[a], no gas produced, no H_2S [18–24 hours]	*Figure 48*
Lysine iron agar (LIA)	K/K (purple slant / purple butt)[a,b], no gas produced, no H_2S [18–24 hours]	–
Gram stain	Small, gram-negative curved rods	–
Wet mount	Small, curved rods with darting motility	–

[a] K= alkaline; A= acid

[b] An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated. An acid reaction (yellow) in the butt indicates that lysine was not decarboxylated.

String test

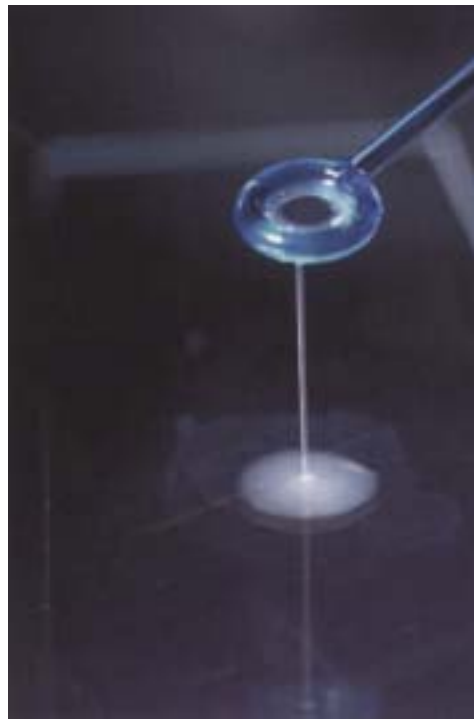
The string test uses fresh growth from nonselective agar and is useful for ruling out non-*Vibrio* species, particularly *Aeromonas* species. The string test may be performed on a glass microscope slide or plastic petri dish by suspending 18- to 24-hour growth from heart infusion agar (or other noninhibitory medium) in a drop of 0.5% aqueous solution of sodium deoxycholate. If the result is positive, the bacterial cells will be lysed by the sodium deoxycholate, the suspension will lose turbidity, and DNA will be released from the lysed cells, causing the mixture to become viscous. A mucoid "string" is formed when an inoculating loop is drawn slowly away from the suspension (Figure 47). *V. cholerae* strains are positive, whereas *Aeromonas* strains are usually negative (Table 19). Other *Vibrio* species may give a positive or weak string test reaction.

Kligler iron agar and triple sugar iron agar

KIA and TSI can be used to rule out *Pseudomonas* species and certain *Enterobacteriaceae*. **It is important that Kligler iron agar and triple sugar iron agar be prepared so the tubes have a deep butt and a long slant;** if the butt is not deep enough, misleading reactions may occur in these media (Appendix 2). A tube prepared so that the butt is approximately 3.5-cm deep and the slant is approximately 2.5-cm is acceptable.

KIA or TSI agar slants are inoculated by stabbing the butt and streaking the surface of the medium. Incubate the slants at 35°–37°C and examine after 18–24 hours. **Caps on all tubes of biochemical media should be loosened before incubation,** but this is particularly important for KIA or TSI slants. **If the caps are too tight**

FIGURE 47: A positive string test with ***Vibrio cholerae***



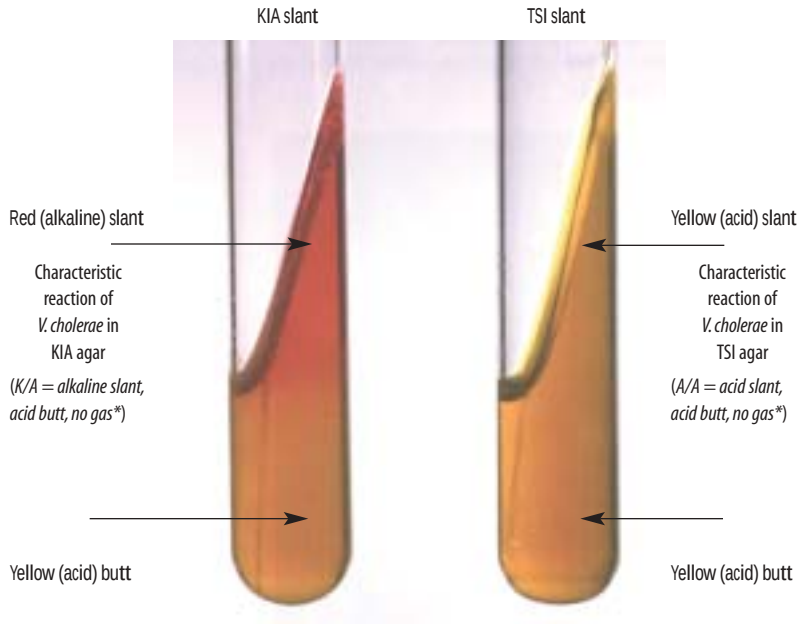
and anaerobic conditions exist in the tube, an inappropriate reaction will occur and the characteristic reactions of *V. cholerae* may not be exhibited.

The reactions of *V. cholerae* on KIA, which contains glucose and lactose, are similar to those of lactose-nonfermenting *Enterobacteriaceae* (*i.e.*, alkaline [red] slant, acid [yellow] butt, no gas, and no H_2S). However, on TSI, *V. cholerae* strains produce an acid (yellow) slant, acid (yellow) butt, no gas, and no H_2S (Table 19 and Figure 48).

Lysine iron agar

LIA is helpful for screening out *Aeromonas* and certain *Vibrio* species, which, unlike *V. cholerae*, do not decarboxylate lysine. LIA must be prepared so that the tubes have a deep butt (approximately 3.5 cm) and a long slant (approximately 2.5 cm). As with KIA and TSI, if the butt is not deep enough, misleading reactions may occur in this medium. In LIA, the decarboxylation of lysine occurs only in anaerobic conditions and a false-negative reaction may result from insufficient medium in the tube (Appendix 2). Inoculate LIA by stabbing the butt and then

FIGURE 48: Reactions of ***Vibrio cholerae*** in Kliger iron agar (KIA) and triple sugar iron agar (TSI)



* Red color indicates an alkaline reaction, and yellow indicates acid. *V. cholerae* does not produce gas or H_2S in either KIA or TSI agar. If gas were present, the agar would appear to be fractured, or cracked. Production of H_2S would be indicated by a blackening of the medium.

streaking the slant; after incubation for 18–24 hours at 35°–37°C, examine the LIA slants for reactions typical of *V. cholerae*. Organisms that produce lysine decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the tube (Figure 41); organisms without the enzyme produce an acid reaction (yellow color) in the butt portion of the tube. H_2S production is indicated by a blackening of the medium. The LIA reaction for *V. cholerae* is typically an alkaline slant (purple), alkaline butt (purple), no gas, and no H_2S (Table 19). *Proteus* and *Providencia* spp. will often produce a red slant caused by deamination of the lysine.

Gram stain

Examining overnight growth of *Vibrio cholerae* from a heart infusion agar slant by Gram stain will demonstrate typical small, curved gram-negative rods (Table 19). Staining with crystal violet only is a more rapid technique that will also demonstrate the cell morphology typical of *Vibrio* species.

Wet mount

Dark-field and phase-contrast microscopy have been used for screening suspected isolates of *V. cholerae*. With these techniques, saline suspensions are microscopically examined for the presence of organisms with typical small, curved rods and darting (*i.e.*, "shooting star") motility (Table 19).

Serologic identification of *V. cholerae* O1 and O139

Following presumptive biochemical identification of the agent as *V. cholerae*, it is appropriate to confirm identification with serology. **If an epidemic of cholera is suspected, the most common cause is *V. cholerae* O1. If *V. cholerae* O1 is not isolated, the laboratory should test for *V. cholerae* O139. If neither of these organisms is isolated, arrangements should be made to send stool specimens to a reference laboratory.** Local and regional laboratories should send isolates requiring testing with O139 antiserum to the national reference laboratory; if the national reference laboratory is still unable to confirm the identification of a *V. cholerae* isolate as O1 or O139, an international reference laboratory can provide guidance.

To conserve resources, the laboratory can first test *V. cholerae* for somatic O1 antigens, and then test with O139 antiserum only if the isolate does not yield a positive agglutination reaction in the O1 antiserum.

Presumptive identification using O1 and O139 antisera

For slide agglutination testing with polyvalent O1 or O139 antisera, fresh growth of suspected *V. cholerae* from a nonselective agar medium should be used. (**Using growth from thiosulfate citrate bile salts sucrose (TCBS) agar may result in false-negative reactions.**) After 5–6 hours of incubation, growth on the surface of the slant is usually sufficient to perform slide agglutination with antisera; if not, incubate for a longer period. If the isolate does not agglutinate in O1 antiserum, test in O139 antiserum. If it is positive in the polyvalent O1 or in the O139 antiserum, the isolate may be reported as presumptive *V. cholerae* O1 or O139. Presumptive *V. cholerae* O1 isolates should be tested in monovalent Ogawa and Inaba antisera (methods follow this section). Once one colony from a plate has been identified as *V. cholerae* O1 or O139, no further colonies from the same plate need to be tested. [Refer to Appendix 2 for a discussion on quality control of antisera.]

Confirmation of V. cholerae *O1 using Inaba and Ogawa antisera*

The O1 serogroup of *V. cholerae* has been further divided into three serotypes: Inaba, Ogawa, and Hikojima (which is very rare). Serotype identification is based on agglutination in monovalent antisera to type-specific O antigens (Table 20). A positive reaction in either Inaba or Ogawa antiserum is sufficient to confirm the identification of a *V. cholerae* O1 isolate. Isolates that agglutinate weakly or slowly with serogroup O1 antiserum but do not agglutinate with either Inaba or Ogawa

antiserum are not considered to be serogroup O1. Identifying these antigens is valid only with serogroup O1 isolates. For this reason, Inaba and Ogawa antisera should never be used with strains that are negative with polyvalent O1 antiserum.

Strains of one serotype frequently produce slow or weak agglutination in antiserum to the other serotype, depending on how well the serotype-specific antisera have been absorbed. For this reason, **agglutination reactions with Inaba and Ogawa antisera should be examined simultaneously, and the strongest and most rapid reaction should be used to identify the serotype.** With adequately absorbed antisera, strains that agglutinate very strongly and equally with both the Ogawa and Inaba antisera are rarely, if ever, encountered. If such reactions are suspected, the strains should be referred to a reference laboratory for further examination and may be referred to as "possible serotype Hikojima."

Slide agglutination procedure

Agglutination tests for *V. cholerae* somatic O antigens may be conducted in a Petri dish or on a clean glass slide.

a) Divide the slide into test sections with a wax pencil and place one small drop of physiological saline in each test section on the slide.

b) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, HIA, or other **non-selective** agar medium. (Serologic testing should not be done on growth from selective media such as MacConkey or XLD agar because selective media may yield false-negative serologic results.) Emulsify the growth in each drop of physiological saline on the slide and mix thoroughly to create a moderately milky suspension.

c) Add a small drop of antiserum to one of the suspensions; the second suspension serves as the control. To conserve antiserum, volumes as small as 10 µl can be used; a bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32). Usually approximately equal volumes of antiserum and growth suspension are mixed, although the volume of suspension may be as much as double the volume of the antiserum.

TABLE 20: Agglutination reactions in absorbed antiserum of serotypes of ***Vibrio cholerae*** serogroup O1

V. cholerae O1 serotype	Ogawa antiserum	Inaba antiserum
Ogawa	+[a]	–[b]
Inaba	–	+
Hikojima[c]	+	+

[a] + indicates a positive agglutination reaction in the absorbed antiserum.

[b] – indicates a negative agglutination reaction in the absorbed antiserum.

[c] If there is a positive reaction in both Ogawa and Inaba antisera and the Hikojima serotype is suspected, send the isolate to an international reference laboratory, following packing regulations as presented in Appendix 12.

d) Mix the suspension and antiserum well and then tilt the slide back and forth to observe for autoagglutination (Figure 2). The agglutination is more visible if the slide is observed under a bright light and over a black background. If the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 42). Examine the saline suspension carefully to ensure that it is even and does not show clumping resulting from autoagglutination. If autoagglutination occurs, the culture is termed "rough" and cannot be serotyped.

Confirmation of V. cholerae *O139*

A suspected *V. cholerae* isolate that reacts in O139 antiserum but not in polyvalent O1 antiserum should be sent to a reference laboratory. Confirmation of *V. cholerae* O139 includes testing for production of cholera enterotoxin and verification of the O139 antigen by slide agglutination with O139 antiserum. No serotypes have been identified in the O139 serogroup. Enterotoxin assays (*e.g.*, PCR, EIA, DNA probing) are complex and beyond the scope of this manual. Few laboratories are capable of doing these tests, and they are performed mainly by international reference laboratories. (See Appendix 12 for packing and shipping regulations and Appendix 14 for a list of international reference laboratories.)

Following identification of the agent, it is appropriate for the laboratorian to commence testing for antimicrobial susceptibility patterns if antimicrobial agents are to be used to treat the cholera outbreak.

Antimicrobial susceptibility testing of *V. cholerae*

As antimicrobial resistance increases in many parts of the world, monitoring the antimicrobial susceptibility of *Vibrio cholerae* O1 and O139 has become increasingly important. The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),[33] and if performed precisely according to the protocol below, will provide data that can reliably predict the in vivo effectiveness of the drug in question. However, **any deviation from the testing method may invalidate the antimicrobial susceptibility test results.** For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for antimicrobial susceptibility testing.

Specific methods for determination of antimicrobial susceptibility patterns of *V. cholerae* are presented in this manual; however, there are some general guidelines that must first be considered before proceeding: test isolates from the beginning of

[33] Formerly known as the "National Committee for Clinical Laboratory Standards," NCCLS is now known solely by its acronym.

an outbreak; test appropriate antimicrobial agents; provide timely feedback to public health officials; and, periodically monitor the epidemic for shifts in antimicrobial susceptibility patterns.

- *Test the isolates from the beginning of the outbreak*
  Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic. That number will provide sufficient information to develop an antimicrobial treatment policy for the organism. After that, the laboratory should conduct periodic surveys to detect any changes in antimicrobial susceptibility patterns. WHO surveillance manuals can provide guidance in the development of such surveys.

- *Test appropriate antimicrobial agents*
  The laboratory should not routinely test antimicrobial agents that are not available in the country or antimicrobial agents that are not recommended by WHO as efficacious in the treatment of cholera (Table 21). In addition, if all isolates are resistant to a particular antimicrobial agent (*e.g.*, to ampicillin) during the first round of testing, testing against those agents during future surveys of the outbreak strain is probably not warranted (although testing of isolates may still be performed once or twice a year to confirm resistance). Sending 10 to 20 of the initial isolates to an international reference laboratory (Appendix 14) can be useful for confirmatory identification of the strain and antimicrobial susceptibility pattern. Guidelines for the packing and shipping of etiologic agents are included in Appendix 12.

- *Provide timely feedback to public health officials*
  Once the organisms are isolated and the antimicrobial susceptibility patterns determined, these results should be transmitted as quickly as possible to the national epidemiologist and to other public health officials. The data can then be used to make rational choices for antimicrobial treatment policy.

- *Monitor for changes in antimicrobial susceptibility*
  As a cholera epidemic progresses, periodic surveys of 30 to 50 isolates of the epidemic organism should be carried out to detect any changes in the antimicrobial susceptibility pattern of the organism causing the epidemic. These surveys should be conducted every 2–6 months, depending on conditions and resources. Any changes should be reported to the national epidemiologist and to other public health officials so that the antimicrobial treatment policy can be modified, if necessary. If any major changes are noted, it is useful to send isolates to an international reference laboratory for confirmation.

The antimicrobial agents recommended by the WHO for testing of *V. cholerae* are included in Table 21; these recommendations are current as of 2002.

In addition to the general principles of antimicrobial susceptibility testing presented in the previous section, there are several special considerations to be heeded when performing disk diffusion testing of *Vibrio cholerae*:

TABLE 21: Antimicrobial agents suggested for use in susceptibility testing of ***Vibrio cholerae*** O1 and O139

Antimicrobial agents for susceptibility testing of ***V. cholerae***
Trimethoprim-sulfamethoxazole (cotrimoxazole)
Furazolidone
Tetracycline[a]
Nalidixic acid[b]

[a] The results from the tetracycline disk are also used to predict susceptibility to doxycycline.

[b] If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.

- Although the disk diffusion technique is the most commonly used method for antimicrobial susceptibility testing, zone size interpretive criteria for *V. cholerae* O1 and O139 have been established by NCCLS only for ampicillin, chloramphenicol, sulfonamides, tetracycline, and trimethoprim-sulfamethoxazole. The interpretations of susceptible, intermediate and resistant for isolates tested against these drugs by disk diffusion correlate well with the minimum inhibitory concentration (MIC) results determined by broth microdilution.
- Disk diffusion tests should not be used for doxycycline and erythromycin because the results for these drugs are frequently inaccurate for *V. cholerae* O1 and O139 strains. Therefore, these agents should not be tested using this method.
- The results from the tetracycline disk should be used to predict susceptibility to doxycycline. If a strain is susceptible to tetracycline, it will also be susceptible to doxycycline.
- At this time there is no *in vitro* method to accurately determine susceptibility to erythromycin.
- The reliability of disk diffusion results for other antimicrobial agents, including ciprofloxacin, furazolidone, and nalidixic acid, has not been validated.
  - Until interpretive criteria have been established for *V. cholerae*, disk diffusion may be used to screen *V. cholerae* for resistance to ciprofloxacin, using NCCLS interpretive criteria for the *Enterobacteriaceae* (Table 22).
  - Tentative breakpoints have been proposed for testing furazolidone and nalidixic acid with *V. cholerae* based on multi-laboratory studies using NCCLS testing methodologies. When screening with the disk diffusion method for these agents, results should be interpreted with caution (Table 22).

Procedure for agar disk diffusion antimicrobial susceptibility testing of *V. cholerae*

Laboratory diagnostic supplies required for *V. cholerae* disk diffusion testing are listed in Appendix 9. Figure 33 summarizes the disk diffusion method of antimicrobial susceptibility testing for enteric bacterial pathogens. The following section provides seven steps for antimicrobial susceptibility testing of *Vibrio cholerae* by the disk diffusion method.

1. ***Mueller-Hinton antimicrobial susceptibility test agar***

   Mueller-Hinton agar medium is the only antimicrobial susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar, poured to a uniform depth of 3–4 mm, should always be used for disk diffusion antimicrobial susceptibility testing, according to NCCLS and international guidelines. Because the way in which Mueller-Hinton is prepared can affect disk diffusion test results, this medium should be prepared strictly according to the methods and quality control instructions presented in Appendix 2.

2. ***McFarland turbidity standard***

   A 0.5 McFarland turbidity standard should be prepared and quality controlled prior to beginning antimicrobial susceptibility testing (Appendix 2, Figure 50). If tightly sealed to prevent evaporation and stored in the dark, the turbidity standard can be stored for up to 6 months. The 0.5 McFarland turbidity standard is used to adjust the turbidity of the inoculum for the antimicrobial susceptibility test.

3. ***Preparation of inoculum***

   Each culture to be tested should be streaked onto a non-inhibitory agar medium (*e.g.*, blood agar, brain heart infusion agar, or tryptone soy agar [TSA]) to obtain isolated colonies. After incubation at 35°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline or nonselective broth (*e.g.*, Mueller-Hinton broth, heart infusion broth, or tryptone soy broth [TSB]) and vortex thoroughly. **The bacterial suspension should then be compared to the 0.5 McFarland turbidity standard.** This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (Figures 51 and 52 in the McFarland turbidity standard section of Appendix 2). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the 0.5 McFarland turbidity standard, the turbidity can be reduced by adding sterile saline or broth, or increased by adding more bacterial growth.

   Alternatively, the growth method may be used to prepare the inoculum. Pick four or five colonies from overnight growth on agar and inoculate them into

broth (Mueller-Hinton broth, heart infusion broth, or TSB). Incubate the broth at 35˚C until turbid (usually 16–24 hours), and then adjust the turbidity to the proper density.

4. ***Inoculation procedure***

   Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 34). Finally, swab around the entire edge of the agar surface.

   If the bacterial colonies used to prepare the suspension are picked off a plate containing mixed growth (*i.e.*, if isolated colonies are picked from a plate that does not contain pure culture), laboratorians may choose to prepare a purity plate to ensure the suspension used for antimicrobial susceptibility testing is pure. To prepare the purity plate, after inoculating the Mueller-Hinton agar plate for confluent growth, label (a portion of) a separate TSA plate (or other non-selective medium) and **use the same swab** of suspension with which the Mueller-Hinton was inoculated to streak for isolation; do not place the swab back into the suspension. Several inocula can be streaked on different sections of a properly labeled purity plate, but the streaks **must not overlap.**

5. ***Antimicrobial disks***

   The working supply of antimicrobial disks should be stored in the refrigerator (at 4˚C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before use.

   Apply the antimicrobial disks to the plates as soon as possible after they are dry, but no longer than 15 minutes after inoculation. Place each disk individually with sterile forceps or with a mechanical dispensing apparatus, and then gently press down onto the agar. In general, **no more than 12 disks are placed on a 150-mm plate and no more than four disks are placed on a 100-mm plate** to prevent overlapping of the zones of inhibition and possible resultant error in measurement. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.** After the disks are placed on the plate, invert the plate and incubate at 35°C for 16–18 hours; if a purity plate was prepared, incubate it under the same conditions.

6. Recording and interpreting results

After incubation, measure and the diameter of the zones of complete inhibition (including the diameter of the disk) in millimeters. (Figure 43 shows growth, Figure 6 and Figure 28 show how to measure the zones, and Figure 49 presents a sample worksheet in which to record data.) The measurements can be made with calipers or a ruler on the under-surface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (approximately 80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. The zones of growth inhibition should be compared with the zone-size interpretative table (Table 22), and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. Measure the distance from the inner-most colonies (*i.e.*, those closest to the disk) to the center of the antimicrobial disk, and double this measurement to obtain the diameter; record the measurement and interpretation of antimicrobial susceptibility (Figure 49). If there is both an inner- and outer zone of inhibition of growth around the antimicrobial disk:

a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)

b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).

c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

The presence of colonies within a zone of inhibition may predict eventual resistance to that antimicrobial agent.

7. Quality control of agar disk diffusion antimicrobial susceptibility testing

To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. (ATCC 25922 is the *E. coli* control strain used when testing Enterobacteriaceae [*e.g., Shigella, Salmonella, Escherichia, Klebsiella*] and *V. cholerae.*) Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits; Table 22 includes the diameters of the zones of inhibition for ATCC 25922. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For

FIGURE 49: Sample form for recording antimicrobial susceptibility results for ***Vibrio cholerae***

Date of Testing: _____/_____/_________

*Test performed by:*_________________________

Interpretation of susceptibility: *S* = susceptible *I* = intermediate *R* = resistant

Specimen number	**Furazolidone** [a]	**Trimethoprim-sulfamethoxazole**	**Tetracycline** [b]	**Nalidixic acid** [a]	*(other drug)* ____________
	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***
	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***
	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***
	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***	mm ***S I R***
E. coli ATCC 25922 (NCCLS QC strain) *QC in range?* →	mm ***Yes No***	mm ***Yes No***	mm ***Yes No***	mm ***Yes No***	mm ***Yes No***

[a] Proposed interpretive criteria based on multi-laboratory studies. (NCCLS criteria have not been established for *V. cholerae* with furazolidone or nalidixic acid.) If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.

[b] Susceptibility of a *V. cholerae* isolate to tetracycline is used to predict susceptibility to doxycycline as well as tetracycline (but doxycycline is not tested directly).

Reviewed by : _____________ ***Date:*** ____/____/________

Note: After 16–18 hours incubation, check the results for the NCCLS-recommended quality control (QC) strain E. coli ATCC 25922 against the acceptable range of inhibition zone diameters and then record disk diffusion results (mm). (Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 22.)

TABLE 22: Interpretive standards for antimicrobial susceptibility testing of ***Vibrio cholerae*** with selected antimicrobial disks

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml)			(NCCLS QC strain ***E. coli*** ATCC 25922
		Susceptible	Intermediate	Resistant	
Ampicillin [a]	10 µg	≥ 17 mm *(≤ 8 µg/ml)*	14 – 16 mm *(16 µg/ml)*	≤ 13 mm *(≥ 32 µg/ml)*	16 – 22 mm *(2–8 µg/ml)*
Chloramphenicol [a,b]	30 µg	≥ 18 mm *(≤8 µg/ml)*	13 – 17 mm *(16 µg/ml)*	≤ 12 mm *(≥ 32 µg/ml)*	21 – 27 mm *(2–8 µg/ml)*
Furazolidone [c]	100 µg	≥ 18 mm	-	< 18 mm	22 – 26 mm [d] -
Nalidixic acid [c]	30 µg	≥ 19 mm	-	< 19 mm	22 – 28 mm *(1–4 µg/ml)*
Ciprofloxacin [e]	5 µg	≥ 21 mm *(≤1 µg/ml)*	16 – 20 mm *(2 µg/ml)*	≤ 15 mm *(≥ 4 µg/ml)*	30 – 40 mm *(0.004–0.016 µg/ml)*
Tetracycline [a]	30 µg	≥ 19 mm *(≤ 4 µg/ml)*	15 – 18 mm *(8 µg/ml)*	≤ 14 mm *(> 16 µg/ml)*	18 – 25 mm *(0.5–2 µg/ml)*
Trimethoprim-sulfamethoxazole [a] (cotrimoxazole)	1.25 / 23.75 µg	≥ 16 mm *(≤ 2/38 µg/ml)*	11 – 15 mm *(4/76 µg/ml)*	≤ 10 mm *(≥ 8/152 µg/ml)*	23 – 29 mm *(≤ 0.5/9.5 µg/ml)*

[a] *Source:* NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.

[b] Use these interpretive standards for chloramphenicol with caution because the disk diffusion test may misclassify many organisms (high minor error rate) [NCCLS 2002].

[c] Proposed interpretative criteria based on multi-laboratory studies; criteria have not been established for *V. cholerae* by NCCLS.

[d] Quality control inhibition zone diameter ranges for furazolidone have not been validated by NCCLS; the ranges presented in this table are based on those suggested by the manufacturer of the antimicrobial disks.

[e] Criteria for interpretation of susceptibility of *V. cholerae* to ciprofloxacin have not been developed; this table presents tentative interpretive criteria based on NCCLS interpretive criteria for *Enterobacteriaceae.*

example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected. For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks even the isolates being tested are susceptible. Again, as mentioned above, erythromycin tested against *V. cholerae* will give

misleading results because these *in vitro* results do not necessarily correlate with *in vivo* activity.

If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease; this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

Data for decision-making: *informed epidemic response*

Once the laboratory has assessed the identity and antimicrobial susceptibility patterns of the *V. cholerae* O1 or O139 isolates, the information should be reported back to public health officials in a timely manner. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent chosen should be effective against at least 80% of local *V. cholerae* O1/O139 strains. Evidence of clinical efficacy is the most important criterion, especially for a drug such as erythromycin, which cannot be tested *in vitro*.
- The antimicrobial agent chosen should be able to be given by mouth.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in manner appropriate to the local situation and antimicrobial susceptibility profile.

CHAPTER X

Conclusion

The techniques and media described in this manual adhere to internationally recognized clinical standards. The procedures provide laboratorians from regions with limited resources with the methodological tools needed for the quality-controlled detection of antimicrobial resistance in seven pathogens causing acute bacterial infections of public health importance. Application of these methods will enable laboratorians to make valid comparisons and interpretations of their findings within countries and across borders.

This manual addresses the identification and antimicrobial susceptibility testing of bacterial pathogens that cause acute respiratory infections, meningitis, febrile illness, diarrheal disease, and sexually transmitted infections of public health concern. *Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae* are three pathogens contributing to a substantial proportion of bacterial pneumonia and meningitis morbidity and mortality; commonly used antimicrobial agents (*e.g.*, penicillin and trimethoprim-sulfamethoxazole) are becoming decreasingly effective for treatment of these pathogens. Laboratory data on antimicrobial susceptibility and serotype (or serogroup) distributions can help determine not only if antibiotic treatment or prophylaxis is appropriate, but also if vaccination would be efficacious. Antimicrobial resistance in *Neisseria gonorrhoeae* is a growing concern not only because of its direct health effects on the reproductive tract, but also because epidemiological evidence indicates that gonorrhea infection facilitates transmission of HIV/AIDS. Typhoid fever, a disease caused by *Salmonella* serotype Typhi, is endemic in many developing countries, and outbreaks of multi-drug resistant strains have been reported worldwide. *Shigella* is frequently the agent of epidemic bloody diarrhea and has become progressively more resistant to commonly available and affordable treatment regimens. Cholera, an internationally reportable disease caused by *Vibrio cholerae* O1 and O139 that is clinically recognized by the presentation of abundant watery diarrhea, must be treated primarily with rehydration therapy, but antimicrobial

agents contribute to the reduction of stool volume. As antimicrobial resistant strains of disease spread throughout communities and more people become infected with less-treatable bacteria, the burden on public health and on social and economic development will continue to grow.

A goal of this manual has been to provide public health reference laboratories with a tool to produce standardized antimicrobial susceptibility test results that can be used for public health decision-making. Individual results of antimicrobial susceptibility tests are important for clinical treatment plans; adequate information must be provided to health-care providers. Laboratorians have the power and responsibility to contribute to the shaping of local policy for prevention, control, and treatment of disease by communicating patterns of a pathogen's antimicrobial susceptibility to public health officials. Concerted public health efforts are needed to reduce the frequency and spectrum of antimicrobial resistant disease in both hospital and community settings.